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(57) Abstract

The present invention is based on the pioneering finding that inhibition of expression of certain genes counteracts the cytokine-induced cell death. As long as these genes function normally, cytokine induces cell death; once the expression of said genes in inhibit, the cytokine-induced cell death is inhibited. It follows therefrom that the normal expression is cytokine-induced cell death. In HeLa cells IFN-y induces a biphasic process, which comprises an initial cytostatic phase and a subsequent cytotoxic phase (programmed cell death). The novel genes discovered in accordance with the present invention were found to affect only the later, cytotoxic phase. These genes, referred to as "DAP" (death-associated protein) genes, include DNA molecules which comprise a coding sequence encoding the expression products of the DAP genes or expression products having a similar biological activity, and are at times collectively referred to as "DAP DNA molecules". The expression products of the DAP DNA molecules will be referred to herein at times collectively as "DAP products".

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Description

TUMOR SUPPRESSOR GENES, PROTEINS ENCODED THEREBY AND USE OF SAID GENES AND PROTEINS

FIELD OF THE INVENTION

The present invention relates to the field of tumor–suppressor genes in general, and cytokine–induced cell death in particular.

BACKGROUND OF THE INVENTION

One of the factors which determines the proliferation state of cells is the balance between the growth–promoting effects of proto–oncogenes, and the growth–constraining effects of tumor–suppressor genes.

One mechanism by which these tumor–suppressor genes exert their growth–constraining effect is by inducing the cell to undergo a physiological type of death. Such a controlled cell death is evident in a multitude of physiological conditions including metamorphosis, synaptogenesis of neurons, death of lymphocytes during receptor repertoire selection, controlled homeostasis in the bone–marrow and other proliferative tissues, and others. Such cell death is regulated by the interaction of the cell with other cells or with cell products, for example through the activity of suitable cytokines.

Genetic mutation that inactivates the suppressor genes, liberate the cell from normal growth constraint imposed by other cells, resulting in an uncontrolled growth of the cell without any relation to external signals. This uncontrolled growth is a step in tumorigenesis.

To date, only a few tumor–suppressor genes have been fully characterized including the *retinoblastoma (Rb)* gene, *p53, DCC, NM23 WT–1, NF–1, APC*, and *ras suppressor* genes. A mutation in either of the above genes, probably in both alleles, which leads to either blockage of expression, or production of a faulty protein, hampers the normal control of growth and viability of cells and may thus give rise to cancer.

Growth–inhibiting cytokines have a double effect on the target cell. They can either inhibit the proliferation of the cell, and/or give rise to cell death. To date, blockage or activation of expression of known tumor–suppressor genes was shown to counteract or enhance, respectively, cytokines' inhibition of cells' growth (reviewed by A. Kimchi, 1992, *J. Cell Biochem.*, 50:1–9) but did not have any effect on the death promoting action of cytokines. For example, the growth inhibitory response to cytokines such as TGF–β, was markedly reduced by the inactivation of the *Rb* gene, or the response to IL–6 was enhanced by introducing activated *p53* genes (Pietenpol *et al.*, 1990, *Cell*, 61:777–785; Levy *et al.*, 1993, *Mol. Cell. Biol.*, 13:7942–7952).

Thioredoxin, a small hydrogen carrier protein, has previously been implicated in the IFN–γ–mediated growth arrest of HeLa cells (Deiss, L.P. and Kimchi, A. (1991) Science 234:117–120).

SUMMARY OF THE INVENTION

In the following, the term *"programmed cell death"* will be used to denote a physiological type of cell death which results from activation of some cellular mechanisms, i.e. death which is controlled by the cell's machinery. Programmed cell death may, for example, be the result of activation of the cell machinery by an external trigger, e.g. a cytokine, which leads to cell death.

The present invention is based on the pioneering finding that inhibition of expression of certain genes counteracts the cytokine–induced cell death. Namely, as long as these genes function normally, cytokine induces cell death; once the expression of said genes is inhibited, the cytokine–induced cell

death is inhibited. It follows therefrom that the normal expression product of these genes is involved in programmed cell death, especially in cytokine-induced cell death. In HeLa cells, IFN-γ induces a biphasic process, which comprises an initial cytostatic phase and a subsequent cytotoxic phase (programmed cell death). The novel genes discovered in accordance with the present invention were found to affect only the later, cytotoxic phase. These genes will be referred to herein as *"DAP* (death-associated protein) *genes"*. DNA molecules comprising a coding sequence encoding the expression products of the DAP genes, or expression products having a similar biological activity, will be referred to herein at times collectively as *"DAP DNA molecules"*. The expression products of the DAP DNA molecules will be referred to herein at times collectively as *"DAP products"*.

According to one aspect of the present invention, to be referred to herein as "*the death-promoting aspect*", the above DAP DNA molecules, expression vectors comprising them, or DAP products are used for promoting death of normal or tumor cells. A particular application of the death-promoting aspect is in therapy of diseases or disorders associated with uncontrolled, pathological cell growth, e.g. cancer, psoriasis, and others. The use of DAP DNA molecules in gene therapy or DAP products if produced extracellularly, in accordance with the death-promoting aspect of the invention, may be in conjunction with cytokines, e.g. IFN-γ.

According to another aspect of the invention, to be referred to herein as "*the death-preventing aspect*", agents which prevent the expression of said DAP DNA molecules, or agents which antagonize, inhibit or neutralize the DAP products, are used for protecting cells from programmed cell death. Examples of possible applications of the death preventing aspect of the invention are in prevention of cell death in various degenerative neurological diseases, such as Alzheimer's disease or Parkinson's disease, which are associated with premature death of particular subsets of neurons; prevention of death of T-cells in AIDS patients, which death resembles programmed cell death; prevention of rejection-associated cell death in transplants which is

believed to result, at least in part, from programmed cell death; protection of normal cells from the cytotoxic effects of certain anti-cancer therapies; etc.

According to a further aspect of the present invention, referred to herein at times as *"the screening aspect"*, DAP DNA molecules are used in order to screen individuals for predisposition to cancer. In accordance with this aspect, the screening is carried out by comparing the sequence of each of the DAP DNA molecules to each of the respective DAP genes in the individual. The absence of a DAP gene, a partial deletion or any other difference in the sequence that indicates a mutation in an essential region, may result in a loss of function and as a consequence a predisposition for cancer. For screening, preferably a battery of different DAP genes may be used.

The DAP genes seem to play an important role in programmed cell death and the inhibition of their expression or neutralization of their expression products protects the cell from cytokine-promoted cell death. Examples of such genes are those whose sequences are depicted in Figs. 6, 8 and 12, or whose partial sequences are depicted in Figs. 13 and 14. The gene for the known protease cathepsin D, whose sequence is depicted in Fig. 15, is also revealed here for the first time as functioning as a DAP gene.

DAP DNA molecules useful in the death-promoting aspect of the invention may have the nucleic acid sequence of the DAP gene or other sequences which encode a product having a similar biological activity to that of the DAP product. Such DAP molecules include DNA molecules having a sequence other than that of the DAP gene but which, owing to the degenerative nature of the genetic code, encode the same protein or polypeptide as that encoded by the DAP gene.

It is well known that it is possible at times to modify a protein by replacing or deleting certain amino acids which are not essential for a certain biological function, or adding amino acids in a region which is not essential for the protein's biological function, without such modification essentially affecting the biological activity of the protein. Thus, a DAP DNA molecule useful in the death promoting aspect of the invention may also have a modified sequence

encoding such a modified protein. The modified sequence has a sequence derived from that of the DAP gene or from that of the above degenerative sequence, in which one or more nucleic acid triplets (in the open reading frame of the sequence), has been added, deleted or replaced, with the protein product encoded thereby retaining the essential biological properties of the DAP product. Furthermore, it is known that at times, fragments of proteins retain the essential biological properties of the parent, unfragmented protein, and accordingly, a DAP DNA molecule useful in the death promoting aspect of the invention may also have a sequence encoding such fragments.

A DNA molecule useful in the death–preventing aspect of the invention may have a sequence which is an antisense sequence to that of the DAP gene, or an antisense sequence to part of the DAP gene, blocking of which is sufficient to inhibit expression of the DAP gene. The part of the gene can be either the coding or the non–coding part of the DAP gene. The mRNA transcripts of the antisense sequences hybridize to the mRNA transcripts of the DAP gene and interfere with the final protein expression. Another DNA molecule useful in the death preventing aspect of the invention is a DNA molecule coding for a modified DAP product which is capable of inhibiting the activities of the unmodified DAP product in a dominant negative manner, such as catalytically inactive kinase (DAP–kinase) or any other modified protein whose presence in the cell interferes with the normal activity of the native protein, for example by producing faulty hetero dimers comprised of modified and unmodified proteins which are inactive and the like.

DNA molecules useful in the screening aspect of the invention comprise the sequence of a DAP gene or a sequence of a fragment thereof. Additionally, also the above antisense DNA sequences may be used in the screening aspect of the invention.

The present invention thus provides a DNA molecule comprising a sequence selected from the group consisting of:

(a) a gene whose expression is necessary for the mediation of the cytokine–induced programmed cell death;

(b) a DNA sequence encoding the same protein or polypeptide encoded by the gene defined in (a);

(c) a modified DNA sequence of (a) or (b) in which one or more nucleic acid triplets has been added, deleted, or replaced, the protein or polypeptide encoded by the modified DNA sequence mediating the cytokine–induced programmed cell death similarly to the protein or polypeptide encoded by the gene as defined under (a) or (b);

(d) fragments of any of the DNA sequences of (a), (b) or (c), encoding a protein or a polypeptide having said biological activity;

(e) a sequence which is an antisense to the entire or part of the DNA molecule under (a) and capable of inhibiting the expression of said gene; and

(f) a modified DNA sequence of (a) or (b) in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the modified sequence having dominant negative effect manifested by the ability of said protein or polypeptide to inhibit said cytokine–induced programmed cell death.

In accordance with a specific embodiment, the present invention provides a DNA molecule comprising a nucleic acid sequence selected from the group consisting of:

(a) a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 160–162 and ending at the triplet 466–468 of the sequence depicted in Fig. 6;

(b) a DNA sequence comprising a coding sequence beginning at nucleic acid triplet at position 287–289 and ending at a triplet at positions 816–818 of the sequence depicted in Fig. 6;

(c) a DNA sequence comprising a coding sequence beginning at nucleic acid triplet at position 337–339 and ending at the triplet at position 4603–4605 of the sequence depicted in Fig. 8;

(d) a DNA sequence comprising a coding sequence beginning at position 74–76 and ending at position 1268–1270 of the sequence depicted in Fig. 12;

(e) a DNA sequence comprising a sequence depicted in Fig. 13;

(f) a DNA sequence comprising a sequence depicted in Fig. 14;

(g) a DNA sequence encoding the same protein or polypeptide encoded by any one of the DNA sequences of (a) – (f);

(h) a DNA sequence as in (a) – (g) in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the sequence having essentially the same biological activity as that encoded by any one of the DNA sequences of (a) – (g), respectively;

(i) fragments of any one of the DNA sequences of (a) – (h), encoding a protein or polypeptide retaining a biological activity present in the protein or polypeptide encoded by any one of the DNA sequences under (a)–(h), respectively;

(j) a sequence which is an antisense to the entire or part of any one of the sequences under (a) – (f) or of the cathepsin D gene and capable of inhibiting the expression of said sequence; and

(k) a modified DNA sequence of any one of the sequences (a) – (f) in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the modified sequence having dominant negative effect and being capable of inhibiting the function of the protein or polypeptide encoded by any one of the sequences (a) – (f), respectively.

The preferred antisense sequences as defined in (j) above are those to the sequences beginning at position 1000 and ending at position 1320 of the DAP–1 gene in Fig. 6, 3781–4148 of the DAP–2 gene in Fig. 8, 74–1270 of the DAP–3 gene in Fig. 12, and 1203–1573 of the cathepsin D gene in Fig. 15.

The present invention also provides a vector comprising any of the above DNA molecules, the vector comprising also sequences required for maintaining and replicating it in a host cell. Vectors in accordance with the present invention may be transfer vectors for propagating and replicating the DNA sequences in a host cell or may be expression vectors comprising also sequences required for translation of said DNA sequences into an mRNA. Examples of such expression vectors are plasmids, e.g. episomes or viruses.

Examples of episomes are those constructed by using the vehicles pTKO1, pTKO2 and pTKO3 (Deiss and Kimchi, *supra*).

The present invention also provides a DAP product which is a protein or polypeptide encoded by a DNA molecule of the invention, with the exception of the DNA molecules having an antisense sequence, or such a protein or polypeptide which has been chemically modified, for example, by methylation, glycosylation, etc. An example of a DAP product is that having the amino acid sequence depicted in Figs. 6, 8 and 12. The DAP product is useful in the death-promoting aspect of the present invention. In accordance with this aspect, the protein may be administered to patients, in particular, to cancer patients, which administration may cause death of the transformed cells.

The present invention further provides agents which inhibit, antagonize or neutralize the DAP product, which are useful in the death-preventing aspect of the invention. Such agents are for example, antibodies directed against the DAP product; inhibitors or antagonists of the DAP product which are able to counteract their effect and prevent the death-promoting activity of the DAP product.

The present invention further provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an active agent being selected from the group consisting of: (i) an expression vector comprising a DNA molecule of the invention or a DNA molecule coding for cathepsin D; (ii) a DAP product of the invention or cathepsin D; and (iii) an antibody, inhibitor or antagonist to the DAP product. The pharmaceutical composition of the present invention may also comprise means for targeting said active agent to the desired cell or tissue. Depending on the nature of the active agent, the composition is useful either in accordance with the death-promoting or the death-preventing aspect of the invention. In accordance with the death-promoting aspect of the invention, the pharmaceutical composition may also comprise a cytokine, e.g. IFN-γ, in combination with a suitable DAP product, or with an expression vector comprising a suitable DAP molecule.

Further provided by the present invention is a method of treatment comprising administering said active agent to an individual. Similarly as in the pharmaceutical composition, depending on the nature of said active agent, the method is practisable in either the death–promoting aspect of the invention or the death–preventing aspect of the invention. In the death–promoting aspect of the invention, said active agent may be administered in conjunction with a cytokine, e.g. IFN–γ.

In accordance with the screening aspect of the invention, there is provided a method for detecting the absence of the DAP gene, a partial deletion or a mutation (i.e. point mutation, deletion or any other mutation) in the DAP genes of an individual, comprising probing genomic DNA or cDNA from the individual with a DNA probe or a multitude of DNA probes having a complete or partial sequence of the DAP genes or having a sequence which is an antisense to the complete or partial sequence of the DAP gene. A particular application of the screening aspect of the invention is in the screening for individuals having a predisposition to cancer, an absence of the gene or a detected mutation or deletion indicating that the individual has such predisposition. The method in accordance with the screening aspect typically comprises the following steps:

(a) obtaining a sample of either genomic DNA from cells of the individual or cDNA produced from mRNA of said cells;

(b) adding one or more DNA probes each of said probes comprising a complete or partial sequence of a DAP gene, or a sequence which is an antisense sequence to the complete or partial sequence of the DAP gene;

(c) providing conditions for hybridization between the DNA probe or probes and the DNA of said sample;

(d) on the basis of the hybridization determining whether the DAP gene is absent or there is a match between the sequence of the DNA probe or probes and a sequence in the DNA of said sample or a mismatch, a mismatch indicating a deletion or a mutation in the genomic DNA and a predisposition to cancer in the tested individual.

A specific embodiment of the screening aspect of the invention involves use of a complete or partial sequence of that shown in Figs. 6, 8, 12–14 or 15, or an antisense of the complete or partial sequence in Figs. 6, 8, 12–14 or 15.

The mutation in the DAP gene indicating a possible predisposition to cancer can also be detected by the aid of appropriate antibodies which are able to distinguish between a mutated and non-functional and a normal functional DAP gene product.

DESCRIPTION OF THE DRAWINGS

Figs. 1 A–D show RNA and protein expression of the DAP-1 gene, wherein:

Fig. 1(A) shows a Northern blot analysis of sense and antisense mRNA obtained from HeLa cells transfected with the constructs 230, 255, 260, 259 and control cells (parental cells) and probed by labeled cDNA fragments from construct 230. Total RNA was prepared from HeLa cells either before (parental) or after transfection with pTKO1 constructs #230 or #255 (group 1), #260 (group 5) and #259 (group 3) designated 230-t1, 255-t1, 260-t1 and 259-t1, respectively. Twenty μg RNA were processed on Northern blots and DNA fragment #230 was used as a probe. The arrows point to the position of sense and antisense RNAs.

Fig. 1(B) shows a Northern blot analysis of sense and antisense mRNA obtained from HeLa cells transfected with control construct (DHFR-t2), 230 construct or control cells (parental) cells treated with (+) or without (-) 750 U/ml of IFN-γ for 24 h. The RNA was extracted from the indicated HeLa cells which were grown for 4 days in the absence (-) or presence (+) of IFN-γ (750 U/ml). The Northern blot containing 20 μg RNA samples was hybridized with the cDNA insert of λ1 phage. The Ethidium Bromide staining of the mRNA samples is shown.

Fig. 1(C) shows an SDS polyacrylamide electrophoresis gel of the expressed protein product of DAP-1 cDNA translated *in vitro* in a reticulocyte

lysate preparation. *In vitro* translation of RNA (0.5 μg) transcribed from the λ1 cDNA (lane 2) and from the subclones p6, p4, p5 and p8 are shown in lanes 3–6, respectively. Lane 1 corresponds to the background obtained in the absence of RNA administration to the reticulocyte lysates. The labeled proteins were fractionated on 12% SDS polyacrylamide gels. The position of the radioactive molecular weight markers (Amersham) is marked. The two translated proteins, the major 15kDa and minor 22kDa proteins, are indicated by arrows.

Fig. 1(D) shows an immunoblot analysis of recombinant and cellular 15kDa DAP–1 protein. Bacterially produced DAP–1 protein (300 ng) and the indicated HeLa cell extracts (350 μg) were fractionated on SDS polyacrylamide gels (12%), blotted to nitrocellulose and reacted with affinity purified antibodies generated against the 15kDa DAP–1. The cells were treated with IFN–γ (750 U/ml) for 4 days before their extraction. The two arrows point to the position of the cellular DAP–1 protein. The antibodies also recognize two non–relevant bands of 60 and 45 kDa that are not modulated by the antisense RNA expression. Quantitation of the reduction in DAP–1 protein was done by densitometric analysis. The calibration of the protein content in each slot was done by referring to the signals of the non–relevant bands. The prestained protein markers (Sigma) are marked.

Figs. 2 A–D show RNA and protein expression of the DAP–2 gene, wherein:

Fig. 2(A) shows a Northern blot analysis of sense and antisense mRNA obtained from two clones of HeLa cells transfected with the control constructs (DHFR–t1 and DHFR–t2) and two clones of cells transfected with the 256 construct (t1 and t2). Total RNA was prepared from the 256–t1 and 256–t2 HeLa cell transfectants either before (0 hours) or at 3 and 24 hours after treatment with IFN–γ (750 U/ml) and 20 μg samples were processed on Northern blots. Fragment #256 was used as a probe. The position of the sense and antisense mRNAs is indicated. The GAPDH mRNA levels were used for the calibration of the RNA amounts in each blot.

In Fig. 2(B) the blot consists of total RNA (20 μg) from K562 cells, parental HeLa cells, the two DHFR–transfected HeLa cell populations and the two HeLa cell populations that were transfected with the pTKO1–256. The blot was hybridized with the cDNA insert of λ29. The Ethidium Bromide staining of the RNA samples is shown.

Fig. 2(C) shows an *in vitro* phosphorylation assay. Cell lysates were prepared from COS–7 cells either before (lane 1) or after transfection with the PECE–FLAG expression vector that carries the coding region of the λ29 cDNA (lane 2). Samples of 400 μg were immunoprecipitated with anti–FLAG™ (M2) monoclonal antibodies (IBI) and subjected to phosphorylation assays.

Fig. 2(D) shows immunoblot analysis of recombinant and cellular DAP–2 protein. The COS–7 cells were transiently transfected with the PECE–FLAG–DAP–2 expression vector. Samples of cell lysates, 100 μg from COS–7 cells and 400 μg from HeLa cells, were fractionated on SDS polyacrylamide gels (7.5%), immunoblotted and reacted with affinity purified polyclonal antibodies raised against the N–terminal DAP–2 peptide. In the lower panel the blot was reacted with monoclonal antibodies against vinculin (Sigma Immunochemicals). Lanes: 1, non–transfected COS–1 cells; 2, transfected COS–1 cells; 3, DHFR–t1 cells; 4, 256–t1 cells; 5, 256–t2 cells. In lane 2 the same 160 kDa protein was also detected with anti–FLAG™ (M2) monoclonal antibodies (IBI) (not shown).

Figs. 3 A–C show morphological features of the cytostatic and cytotoxic responses to IFN–γ in HeLa cells. All cultures were seeded at an initial density of 10,000 cells per cm^2.

Fig. 3(A) shows light microscopy of HeLa cells transfected with pTKO1–DHFR construct (DHFR–t1 cells), on days 3 and 8 of culturing in the absence (a,c) or the presence (b,d) of IFN–γ (750 U/ml). (Magnification x 400). Note the absence of refractile mitotic cells during the cytostatic phase of responses to IFN–γ (in b) and the appearance of round cells that were detached from the substratum during the killing phase (in d).

Fig. 3(B) shows staining of DNA with DAPI; a. DHFR–t1 non–treated cells removed by trypsinization and mounted on glass slides. b. Detached DHFR–t1 cells collected 7 days after IFN–γ treatment. Nuclei with condensed or fragmented chromatin are indicated by arrows. (Magnification x 1000).

Fig. 3(C) shows scanning and transmission electron micrographs of cells transfected with the control construct DHFR–t1 and the 230–t1 construct. DHFR–t1 HeLa cell populations (a–d) and the 230–t1 antisense transfected cells (e and f), were cultured either in the absence (a, c, e) or in the presence (b, d, f) of IFN–γ (750 U/ml). (a,b,e,f), scanning electron micrographs were taken after 7 days using GSM 6400 SEM (Jeol). Bars=10 mm (x2200 magnitude in all the four samples). (c and d), transmission electron micro–graphs taken after 7 days using TEM (Philips 410) at a magnitude of x2800. The condensed nuclei and the surface blebs are indicated by arrows.

Figs. 4 A–C show that the antisense RNA expression from plasmids of groups 1 and 2 reduces the susceptibility of HeLa cells to the killing effects of IFN–γ but has no effect on early IFN–γ signalling.

Figs. 4 (A–B) show the number of viable cells as determined by light absorption at 540 nm, as a function of time; the cells being transfected either with the control construct DHFR–t1 (● – 1(A) and 1(B)); the 255 or 230 construct (▲ – 1(A)) or with two clones t1 and t2 of the 256 construct (▲ – 1(B)). The results are shown both for cell growth with (+) and without (–) administration of 750 U/ml of IFN–γ. Each point is the average of a quadruplicate determination with a SD that ranged between 2–5%.

Fig. 4(C) shows a Northern blot analysis of 2–5A synthetase gene induction. The indicated HeLa cell transfectants were incubated for 24 hours in the presence (+) or absence (–) of IFN–γ (750 U/ml). Twenty mg of total RNA were analyzed. The cDNA of the 2–5A synthetase was used as probe.

Fig. 5 shows the restriction map of the λ1 cDNA clone that carries the DAP–1 cDNA.

Fig. 6 shows the DNA sequence and predicted amino acid sequence of DAP–1.

Fig. 7 shows the restriction map of the λ29 cDNA clone, that carries the DAP–2 cDNA.

Fig. 8 shows the DNA sequence and predicted amino acid sequence of DAP–2.

Figs. 9 A–C show DAP–2 sequence homologies to other serine/threonine kinases and alignment of the ankyrin repeats of DAP–2, wherein:

In **Fig. 9(A)** the protein kinase domain sequences of the DAP–2 are aligned with the corresponding domains of other calmodulin–dependent kinases. The kinase subdomain structure (numbered I–XI) and the region implicated in calmodulin recognition and binding (designated as calmodulin regulatory region) are indicated. The obligatory conserved amino acids within the kinase domain are labeled with asterisks. Numbers at the right mark positions relative to the N–terminus of primary translational products of each kinase. Solid background indicates identical amino acids within the compared kinases. Stippled background indicates positions where the amino acids are not identical but similar. nm–mlck – non–muscle myosin light chain kinase (chicken); sm–mlck – smooth muscle myosin light chain kinase (chicken); skm–mlck – skeletal muscle myosin light chain kinase (rat); camdk–alph,–beta,–gamm – calcium/calmodulin dependent protein kinase II – α–, ß– and γ– subunits, respectively; mlck–dicdi – dictyostelium discoidium (slime mold) myosin light chain kinase.

Fig. 9(B) shows alignment of kinase subdomains II and III of DAP–2 and the corresponding domains of different cell cycle dependent kinases. dm2 – Drosophila CDC2 homologue; pssalre – Human serine/threonine kinase PSSALRE; kpt2 – Human serine/threonine protein kinase PCTAIRE–2; kin28 – yeast (*S.cerevisiae*) putative protein kinase; mo15 – Xenopus protein kinase related to cdc2 that is a negative regulator of meiotic maturation; kkialre – human serine/threonine protein kinase KKIALRE.

Fig. 9(C) shows alignment of DAP–2 ankyrin repeats. Solid background indicates identical amino acids. A consensus sequence of the DAP–2 ankyrin

repeats is shown at the bottom. The position of each individual repeat along the cDNA is illustrated in Fig. 9(B). ar 1–8, ankyrin repeats.

Fig. 10 shows Northern blot analysis of mRNA obtained from several hematopoietic cells probed with labeled DAP–1 cDNA.

Fig. 11 shows Northern blot analysis of mRNA obtained from liver, spleen or brain of normal embryos (2) and embryos with Down Syndrome (1) both probed with the labeled cDNA or DAP–1 or DAP–2. In order to evaluate levels of total mRNA, GAPDH was used (bottom).

Fig. 12 shows the DNA sequence and predicted amino acid sequence of DAP–3.

Fig. 13 shows a partial DNA sequence of DAP–4.

Fig. 14 shows a partial DNA sequence of DAP–5.

Fig. 15 shows the DNA sequence and amino acid sequence of cathepsin D.

DETAILED DESCRIPTION OF THE INVENTION

I. Isolation of antisense cDNA's that protect cells from the cytotoxic effects of IFN–γ

(A) Experimental procedure

(A_1) Obtaining cDNA clones

A cDNA library (100μg DNA) was generated from a mixture of mRNA's harvested before and at 1, 2, 4, 12, 24 and 48 hours after treatment of HeLa cells with IFN–γ (200 U/ml). It was cloned in antisense orientation into the EBV–based pTKO1 expression vector, as previously described in detail (Deiss and Kimchi, *supra*). The resulting expression library of about 10^5 independent clones was introduced into $8X10^6$ HeLa cells (10^6 cells per 9 cm plate) by the calcium phosphate transfection technique. In order to determine the efficacy of transfection, a fraction of the transfectants was selected with hygromycin B (200 μg/ml, Calbiochem). The resulted efficacy was around 5%. In parallel, the majority of the transfected cells were plated at a cell density of 1500 cells per cm^2 and were selected with both hygromycin B (200 μg/ml) and

IFN−γ (750 U/ml). Selective media was changed every 3–4 days. After 28 days the cells that survived and/or grew in the presence of IFN−γ were expanded for 2 weeks and pooled. The extrachromosal DNA was obtained according to the method of Hirt (Hirt, B. (1967) *J. Mol. Biol.*, 26:365), cleaved with the restriction enzyme DpnI and introduced into *Escherichia coli* HB101 host cells. The cleavage with DpnI ensured that only episomal DNA that have replicated in HeLa cells was transfected into bacteria.

A few bacterial clones were obtained by the above procedure which included DNA antisense sequences, some of which were able to protect the cells from the death–promoting effects of IFN−γ.

(A_2) Classification of the antisense cDNA clones

Plasmid DNAs were prepared from 10 individual bacterial clones. PCR amplified cDNA inserts were generated from each plasmid using specific primers that correspond to the immediate flanking sequence of the cDNA insertion sites in the pTKO1 vector. The size of the cDNA inserts ranged between 300 to 800 bp. The PCR fragments were used as labeled probes to search on Southern blots for possible cross hybridization between some of the rescued antisense cDNA clones.

(B) Results

(B_1) Classification of Clones

The above 10 cDNA clones were classified into six distinct non–overlapping groups, some constituting several members (clones) and some constituting of a single member. Those clones relevant for the present invention are shown in the following Table 1:

Table 1: Initial characterization of antisense cDNA clones rescued from IFN−γ−treated HeLa cells

No.	antisense cDNA clones	cDNA length (bp)	mRNA size (Kb)	DNA product
1.	230,254,255,264, 258	320	2.4	DAP−1
2.	256	367	6.3	DAP−2 (kinase)
3.	259	252	1.7	DAP−3
4.	253	~ 300	4.5	DAP−4
5.	260	~ 800	4.0	DAP−5
6.	229	370	2.5	Cathepsin D

Inserts 230, 254, 255, 264 and 258 of group 1 seemed to be completely identical to one another. The PCR fragments were sequenced and the results were compared with sequences present in the EMBA nucleic acid database. All inserts of groups 1 through 5 were found to be novel.

(B_2) Detection of mRNA

The DNA fragments thus obtained were used to detect and determine the expression level in HeLa cells of mRNA which hybridized to these fragments. 20 μg of total RNA from the parental HeLa cells were fractionated on gels, blotted and reacted with the different probes. Each probe recognized a single mRNA transcript of a different size (Table 1). Expression levels of mRNA's reactive with group 2 were low while those reactive with group 1 were relatively high.

II. Second transfection by isolated antisense cDNA

Levels of expression of antisense RNA in secondary transfectants

(A) Experimental procedure

To ensure that the above isolated antisense cDNA's are sufficient in order to protect cells from the death promoting effects of IFN−γ, subconfluent monolayers of HeLa cells were transfected with 40 μg DNA of the individual

rescued pTKO1 plasmids (in duplicates) and subjected to the single selection of hygromycin B. Pools of approximately 10^4 hygromycin resistant clones were generated from each transfection and were kept as 6 duplicates of stable polyclonal populations. The sensitivities of the above clones to an application of IFN−γ was then determined.

The vector pTKO1−DHFR (Deiss and Kimchi, *supra*) which carried a non−relevant construct served as control. The control vector was introduced in parallel into HeLa cells and produced two independent polyclonal population of stable transfectants designated DHFR−t1 and t2.

The double stranded cDNA fragments from construct 230 and 256 (from groups 1 and 2, respectively) were used as probes in Northern blot analysis in order to detect mRNA transcripts both in non−transfected and transfected HeLa cells. These two specific cDNA inserts were labelled by commonly used commercial labelling kits. They were subcloned into Bluescript™ vectors (Stratagene, USA) to facilitate both the preparation of the cDNA inserts and the production of single stranded RNA probes therefrom.

(B) <u>Results</u>

<u>Constructs 230 (group 1)</u>

As can be seen in Fig. 1A the cDNA insert in this construct hybridized to a single endogenous 2.4 Kb mRNA transcript, both in non−transfected and transfected HeLA cells. In stable transfectants containing the antisense constructs of clones 230 and 255, an additional composite antisense transcript was detected by this 230 probe. It consisted of 320 bases of the original cDNA insert and 800 additional bases of sequences derived from the expression cassette (SV40 early promoter together with sequences till the polyadenylation signal). One of the RNA labeled strands produced by the Bluescript™ vector hybridized exclusively to the endogenous 2.4 Kb mRNA while the complementary strand hybridized only to the 1.1 Kb RNA confirming that the latter is indeed an antisense mRNA (data not shown).

The amount of the antisense RNA in clones 230 and 255 exceeded the sense mRNA levels by 3 to 6 fold (Figs. 1A, 1B). After IFN−γ treatment

the level of antisense expression was further elevated due to the presence of IFN−γ−stimulated response element (ISRE) in the pTKO1 vector (Deiss and Kimchi, *supra*), thus leading to 15 fold excess of antisense over sense transcripts (Fig. 1B). The endogenous 2.4 Kb mRNA level was neither modulated by IFN−γ, nor influenced by the high antisense expression.

Construct 256 (group 2)

As can be seen in Figs. 2A and 2B, the construct of the 256 clone (367 bp in size) hybridized on Northern blots to a single endogenous 6.3 Kb mRNA transcript which was expressed in all tested cells at relatively low levels. In the 256−t1 and t2 transfected cells it also hybridized to a composite 1.2 Kb RNA that consisted of 367 bases of the cDNA insert and 800 bases of sequences derived from the expression cassette in the vector (Fig. 2). The antisense orientation of fragment #256 in the pTKO1 vector was confirmed upon sequencing of the sense cDNA clone (Fig. 7). The amount of the antisense RNA expressed from pTKO−1 plasmid #256 in non−treated HeLa cells exceeded the sense mRNA levels by more than 100 fold. Moreover, due to the presence of IFN−stimulated response element (ISRE) in the pTKO1 vector, the levels of antisense mRNA expression were further elevated after IFN−γ treatment (Fig. 3).

III. Response of cells transfected with antisense cDNAs to IFN−γ

(A) Experimental procedure

The HeLa polyclonal population transfected with the individual antisense cDNAs were cultured in the presence of both hygromycin B and IFN−γ (750 U/ml). Growth and viability parameters were examined: (1) under the light microscope, (2) by electron microscopy, and (3) by DAPI staining (0.5 μg/ml; Sigma). For more detailed quantitation, a neutral red uptake assay was performed: the different polyclonal HeLa cell populations were cultivated in 96−well microtiter plates at subconfluent cell densities and then treated with IFN−γ (750 U/ml) or left untreated. All the cells were continuously maintained in a hygromycin B−containing medium to select for transfected

cells. The two DHFR–transfected HeLa cell populations (t1, t2), prepared as described above, served as control cultures that display the typical growth sensitivity curves to IFN–γ. The examined antisense cDNA transfected cells were the 230–t1, 255–t1 (group 1) and 256–t1, 256–t2 (group 2). Viable cells were stained with neutral–red and the dye uptake was quantified by measuring O.D. at 540 nm in quadruplicates during the 14 days of the experiment.

(B) Results

The microscopic examination of parental and control DHFR–transfected HeLa cells revealed that IFN–γ triggered a biphasic pattern of responses. The cells stopped proliferating during the first four days of IFN–γ treatment but still remained viable (in trypan–blue exclusion tests) and displayed a flattened morphology characteristic of the cystostatic responses to IFN–γ (Fig. 3A, b). The reduction in the proliferation rate during this period was also measured by a sharp decline (by more than 90%) in the thymidine uptake into DNA (not shown). This type of IFN–γ–induced proliferation arrest was then followed by massive cell death that occurred in a non–synchronous fashion over a period of an additional 10 days. The cells gradually reduced their size, rounded up and detached from the plates (Figs. 3A, d). Staining of DNA with DAPI after detachment of cells from the substratum revealed gross changes in the nuclear morphology characteristic of programmed cell death. This included nuclear pyknosis, chromatin condensation, sometimes detected preferentially at the nuclear periphery, and chromatin segmentation (Fig. 3B, b). Transmission electron micrographs of the IFN–γ–treated cells prior to their detachment revealed other morphological changes including the disappearance of surface microvilli, surface blebbing, budding off cytoplasmic projections and cytoplasmic disintegration, in addition to the nuclear pyknosis and chromatin condensation (details shown in Fig. 3C, d). The antisense RNA expression from pTKO–1 plasmid of group 1 reduced the susceptibility of the cells to the killing effects of IFN–γ: more cells survived on the plates and the above–mentioned death associated morphological changes appeared at much lower frequency (compare the scanning electron micrographs of the IFN–γ–treated

DHFR–transfected cells in Fig. 3C, b to the IFN–γ–treated 230–t1 cells in Fig. 3C, f). Similar microscopic observations, showing protection from the IFN–γ–induced cell death, were also made with respect to three other clones from the aforementioned groups of antisense cDNAs, i.e. 2, 3, and 7 (not shown).

A neutral–red uptake assay was then performed to determine more accurately, on a quantitative basis, both the typical biphasic responses of control cultures to IFN–γ and the reduced susceptibility of the antisense expressing cultures to the IFN–γ–induced cell death. The two DHFR–transfected HeLa cell populations (t1, t2) served as the control cultures in this assay and the antisense cDNA transfected cells examined were the 230–t1, 255–t1 (group 1) (Fig. 4A) and 256–t1, 256–t2 (group 2) (Fig. 4B). In the absence of IFN–γ, all the transfected HeLa cells behaved the same and displayed practically identical growth curves suggesting that the antisense RNA expression had no effects on the normal growth of cells. Another feature that was not changed by the antisense RNA expression was the extent of the cytostatic responses to IFN–γ. As shown in Figs. 4A and 4B, IFN–γ has similarly reduced the proliferation rate of all the transfected cultures and they all displayed the same extent of reduction in the neutral–red dye uptake during the first 4 days (before cell death starts to be microscopically evident). After 4 days of treatment the picture changed drastically. While almost all control cells died during the subsequent days of IFN–γ treatment leading to minimal values of the neutral–red dye uptake on day 14, a significant fraction of cells that expressed antisense RNA survived in the presence of IFN–γ, as reflected by the sustained values of the dye uptake. The resistance to the IFN–γ–induced cell killing was very similar in all the four tested cultures that expressed the two different antisense RNAs (Figs. 4A, 4B). These data indicate that expression of antisense RNA from groups 1 and 2 protects the HeLa cells exclusively from the IFN–γ–induced cell death and not from its cytostatic action. It is noteworthy that the antisense RNA expression did not affect the early biochemical steps in the signaling of IFN–γ as deduced from the normal mRNA induction by IFN–γ

of the 2–5A synthetase gene in these transfected cells (Fig. 4C). Altogether, it is concluded that among all criteria tested only the death inducing effects of IFN–γ were interrupted by the antisense RNA expression.

IV. Responses of cells transfected with antisense constructs to necrotic cell death

It became interesting at this stage to check whether the antisense RNA expression can also protect the HeLa cells from a necrotic type of cell death. For this, the effect of TNF–α added in combination with cycloheximide (CHX) was examined in the various HeLa cell populations. Unlike the effect of IFN–γ, the cell death that was induced by TNF–α + CHX in HeLa cells was very rapid (50% killing after 3 hours) and displayed typical features of necrosis such as swelling of the cells before their lysis. As shown in Table 2, while the antisense RNA expression from groups 1 and 2 protected the cells from the IFN–γ–induced cell killing, there was no protection from the TNF–α–induced necrotic cell death. All the examined HeLa cell transfectants were killed by the TNF + CHX combination with similar time kinetics and at the same efficiency. Northern blot analysis demonstrated that the levels of the antisense mRNA transcripts in 256–t1 cells were not reduced by the TNF + CHX treatment at 5 hours (not shown) thus excluding the possibility that loss of the antisense RNA expression, caused by the treatment, may be the reason for lack of protective effects from the necrotic cell death. This further suggests a certain specificity of the protective mechanisms regarding the type of cell killing.

Table 2: Expression of antisense RNA (from groups 1 and 2) protects from the IFN−γ−induced programmed cell death but not from the TNF−induced necrotic cell death. (A=540 nm)

		DHFR −t1	DHFR− t2	230−t1	255−t1	256−t1
14 days	No treatment	0.396	0.345	0.385	0.324	0.336
	IFN−γ	0.026	0.017	0.136	0.158	0.159
5 hours	No treatment	N.D.	0.148	0.130	N.D.	0.140
	TNF−α + CHX	N.D.	0.053	0.026	N.D.	0.022
20 hours	No treatment	0.211	0.248	0.223	0.173	0.190
	TNF−α + CHX	0.002	0.001	0.003	0.0015	0.002

Each treatment was done in quadruplicates and the average values of dye uptake, measured by the OD at 1=540 nm, is presented at the indicated time intervals. The SD was between 2−4%. N.D, not done.

V. Cloning of DAP−1 cDNA and determination of amino acid sequence.

An HL−60 cDNA library constructed in λgt10 vector was screened with the cDNA insert of pTKO1−230. Two independent clones, λ1 and λ2, almost completely overlapping and carrying cDNA inserts of about 2.3 Kb were analysed. λ1 cDNA clone encompasses the 5'−untranslated region, short coding region(s) and a relatively long 3'−untranslated region that constitutes more than 60% of the cDNA clone (Fig. 5).

The nucleotide sequence of the cDNA carried by λ1 and its predicted amino acid pattern are presented in Fig. 6. This cDNA is 2232 bp long and contains a potential polyadenylation signal ATTAAA at its 3' end. The open reading frame (ORF) is very short, starting from the initiation codon at nucleotide positions 160−162 and ending at termination codon TGA at positions 466−468. This ORF is preceded by an extremely GC−rich 5'−untranslated region and potentially codes for a protein consisting of 102 amino acids with calculated MW of 11.2 kDa. The amino acid composition predicts

a basic protein (isoelectric point = 10), rich in prolines (15%) which displays two blocks of charged residues, one in the middle and the other at the 3' end of the protein. The high proline content may cause some anomalies in the protein's migration on gels. Search for motifs ("Motifs" program; GCG Software Package) indicated that the protein contains two potential sites for casein kinase II phosphorylation at positions 3 and 36, a single potential protein kinase C phosphorylation site at the C–terminus (position 91) and a consensus phosphorylation site of the cdks at position 51. In addition, the protein contains the consensus sequence RGD at position 65–67, a tripeptide that in some proteins plays a role in cell adhesion, and a potential SH3 binding motif, SPSPP, at position 49–53 (Cowburn (1994) Struc. Biol. 1, 489–491). No indications for the presence of signal peptide or transmembranal domain have been found (SAPS prediction; Brendel et al., (1992) PNAS USA, 89:2002–2006). The amino acid sequence showed no significant homology to known proteins.

Fragment #230 was used as a probe on Southern blots containing human genomic DNA, digested with various restriction enzymes that do not cut it. A single band was visualized upon hybridization with DNA cleaved with EcoRI, BamHI, PstI and XbaI, suggesting the existence of a single copy gene (not shown). This new gene was termed DAP–1 (Death Associated Protein–1).

In vitro translation assays in reticulocyte lysates confirmed that the predicted ORF codes for the major 15kDa protein translated from the cloned 2.4 Kb transcript. The full length cDNA insert as well as four subclones that span different regions of the molecule (i.e., p6, p5, p8, and p4; see Fig. 5) were transcribed and translated in vitro. Among all the tested subclones, only the 5' 1 Kb portion of the DAP–1 cDNA (p6) directed the *in vitro* synthesis of proteins (Fig. 1C). The major translated product migrated on gels as a 15 kDa protein. Mutation at the ATG codon at position 160–162 (ATG to GGC) completely eliminated the synthesis of the 15 kDa protein, thus confirming the position of the start point of this protein (data not shown). In addition to the 15 kDa protein product, a second protein of 22 kDa was also translated at lower

efficiency from λ1 and the p6 cDNAs (Fig . 1C). Its translation was not influenced by the elimination of the ATG codon at position 160 but the protein was shortened to a size of 16 and 18 kDa upon cleavage of the p6 subclone with DraI and BstYI restriction endonucleases, respectively (not shown; for restriction map see Fig. 5). These criteria fit another potential open reading frame, which is detected in the nucleotide sequence in a different phase with respect to the first ORF (Fig. 6). It starts at the ATG codon (positions 287–289) and ends at termination codon TGA (positions 816–818). It has the potential to code for a protein consisting of 176 amino acids with a calculated molecular weight of 19.9 kDa, and has no significant homology to any known proteins.

To analyse the expression of the major DAP–1 protein in cells, rabbit polyclonal antibodies were prepared against the bacterially produced 15kDa protein. The affinity purified antibodies recognized on immunoblots two closely migrating proteins in extracts of HeLa cells; the lower band co–migrated on gels with the bacterially produced 15 kDa DAP–1 protein. The slower migrating form may represent a post–translationally modified version of the protein. In the HeLa cell transfectants, 230–t1 and 255–t1, expressing the elevated levels of antisense RNA that develop in the presence of IFN–γ (15 to 1 ratio), the DAP–1 protein levels were reduced by 75% and 78%, respectively, as compared to the DHFR–tranfected cultures (Fig. 1D). The two upper non specific bands (that are not competed with excess of the bacterially produced DAP–1) were not affected by the antisense expression, thus supporting the selectivity of the effect.

VI. Cloning of DAP–2 and determination of amino acid sequence

As mentioned above, expression studies indicated that the double–stranded cDNA fragment #256 (367 bp in size) hybridized on Northern blots to an endogenous 6.3 Kb mRNA transcript. The same single 6.3Kb mRNA transcript was detected in HeLa (parental and transfectants) and in K562 cells when the full length cDNA (see below) was used as a probe on Northern blots

(Fig. 2B). The cDNA insert from pTKO1–256 was therefore used to screen a K562 cDNA library.

Approximately 4x10^6 pfu were screened with the #256 cDNA insert and 40 positive clones were isolated after two rounds of sequential walking screening. The sequencing was performed on an Applied Bio–systems DNA sequencer 373 A. Sequence uniqueness and relatedness were determined using FASTA (GCG software package) at the nucleotide level and FASTA, BLASTP, and BLOCKS programs at the amino acid level (S. Henikoff and J. G. Henikoff, Nucleic Acids Res. 19, 6565 (1991).

Two clones, λ29 and λ32, were chosen for sequencing (Fig. 7). The resulting composite sequence of both cDNAs consists of 5886 nucleotides and contains a poly A tail that starts at position 5872 and is preceded by two polyadenylation signals AATAAA (Fig. 8). The 3'–untranslated region also contains two ATTTA instability motifs found in the 3'–noncoding portions of short–lived mRNAs (G. Shaw and R. Kamen, Cell 46, 659 (1986)). The mRNA contains a single long open reading frame that starts at position 337, ends at position 4605 and potentially codes for a protein of 1423 amino acids (Fig. 8). The calculated molecular weight of the protein product is about 160 kDa. Affinity purified polyclonal antibodies were raised against the N–terminal 20 amino acid peptide of the protein. These antibodies recognized on immunoblots a 160kDa recombinant protein that was produced in COS–1 cells after transfection with a vector that expressed the entire coding region of the cDNA (Fig. 2D). These antibodies reacted in HeLa cells with an endogenous protein of the same size. In the antisense RNA expressing cells, 256–t1 and 256–t2, the steady state levels of the 160kDa protein were 10 and 5 fold lower than in the DHFR control cells while a non relevant protein, vinculin, displayed similar expression levels in all HeLa cell transfectants (Fig. 2D). Thus, expression of anti–sense RNA from pTKO–1 plasmid #256 in HeLa cells resulted in a significant reduction in the amount of the corresponding protein.

We were able to define several known domains and motifs that are present in this protein. Its extreme N–terminus is composed of a protein kinase

domain that spans 255 amino acids from position 13–267. On the basis of its structure, it is likely to be a serine/threonine type of protein kinase having a classical composition of XI subdomains with all conserved motifs present (Fig. 8) (S. K. Hanks and A. M. Quinn, Methods Enzymol. 200, 38 (1991)). This novel kinase was termed DAP–2 or DAP–kinase (Death Associated Protein–kinase).

The kinase domain falls into a family of that of calmodulin–dependent kinases. The homology to known kinase domains that constitute this group, including the myosin light chain kinases, ranges between 34%–49% (Fig. 9A). Three main differences distinguish the kinase domain of DAP–kinase from other members of calmodulin–dependent kinase family: 1) Subdomain II is relatively long and has a stretch of basic amino acids (KKRRTKSSRR); 2) Subdomain III mostly resembles that of the cell cycle dependent kinases (Fig. 9B). Interestingly, the typical sequences of the cell cycle dependent kinases (PSTAIRE, PSSALRE, PCTAIRE, KKIALRE) are located in subdomain III; and 3) Subdomain VII is extremely short and consists of only 7 amino acids.

Right downstream to the kinase domain there is an additional stretch of homology that is present in almost all members of the family of calmodulin–dependent kinases, and was implicated in calmodulin–recognition and binding; B. P. Herring, J. T. Stull, P. J. Gallagher, J. Biol. Chem. 265, 1724 (1990); M. O. Shoemaker *et al.*, J.Cell. Biol. 111, 1107 (1990); F.H. Cruzalegui *et al.*, Proc. Nath. Acad. Sci. USA 89, 12127 (1992)). Downstream of the calmodulin–recognition domain, an ankyrin repeats domain was identified spanning 265 amino acids from position 365 to 629. It is composed of 8 repeats of 33 amino acids each, not separated by spacers except for a single proline residue that separates three N–terminal repeats from five C–terminal ones (Figs. 8 and 9C). Ankyrin repeats are involved in protein–protein interactions in a variety of proteins (P. Michaely and V. Bennett, Trends in Cell Biology 2, 127 (1992)), but were not described before in the context of serine/threonine kinases. One tyrosine kinase carrying ankyrin repeats has been recently identified in Hydra vulgaris (T.A. Chan *et al.*, Oncogene 9, 1253 (1994)). In

the DAP–kinase, the 8 ankyrin repeats may mediate the interaction with a putative effector or a regulatory molecule, or influence the substrate selectivity and/or stability of the kinase–substrate interactions.

Immediately downstream to ankyrin repeats there are two subsequent potential P–loop motifs, ALTTDGKT and GHSGSGKT, identified through the consensus sequence, G[A]XXXXGKT[S]. Comparison of DAP–kinase potential P–loop motifs to the corresponding consensus sequences within seven ATP or GTP–binding protein families demonstrates that only the 3' P–loop has some similarity to P–loop consensus of elongation factors, ATP synthase b–subunits and thymidine kinase. Actually, a stretch of 33 amino acids following the eighth ankyrin repeat that encompasses the putative 5' P–loop, may represent a ninth ankyrin repeat that is less conserved than others. DAP–kinase also carries multiple potential sites for post–translational modifications, and has neither transmembranal domain nor signal peptide. The Prosite bank search, using the program Motifs (GCG Software Package) revealed that the DAP–kinase protein contains a consensus sequence for the C–terminal amidation site at position 1376 (this suggests that 47 C–terminal amino acids can be cleaved from the protein body). It also contains consensus sequences for six N–glycosylation sites, and potential phosphorylation sites for cAMP–dependent kinase (six), casein kinase II (twenty eight) and protein kinase C (twenty).

Altogether, the deduced amino acid sequence of the DAP–kinase suggests that a very unique type of calmodulin–regulated serine/threonine kinase has been rescued. The combination of serine/threonine kinase domain, ankyrin repeats and additional possible ATP/GTP binding sites outside the kinase domain in one protein (Fig. 10) has not been previously described. A size of 160 kDa is rare among serine/threonine kinases and DAP–kinase is actually the largest calmodulin–dependent kinase known to date. The ability of DAP–kinase to bind calmodulin, recently confirmed in yeast two hybrid system (not shown), is consistent with the notion that in many cases programmed cell death is Ca^{2+} dependent (S. Sen, Biol. Rev.

Camb. Philos. Soc. 67, 287 (1992); S. Lee, S. Christakos, M. B. Small, Curr. Opin. Cell. Biol. 5, 286 (1993)). Moreover, it has been recently reported that calmodulin antagonists inhibited the glucocorticoid-induced apoptosis (D. R. Dowd, D. P. Mac, B. S. Komm, M. R. Haussler, R. Miesfeld, J. Biol. Chem. 266, 18423 (1991)), and that inhibitors of myosin light chain kinases blocked the TNF-induced apoptotic cell death (S.C. Wright, H. Zheng, J. Zhong, F.M.Torti, J.W. Larrick, J. Cell. Biochem. 53, 222 (1993)).

In order to verify that DAP-2 is truely a kinase, COS cells were transiently transfected with an expression vector (PECE-FLAG) that carries a fragment of the I29 cDNA that encompasses the entire coding region (from the abovementioned start ATG to the first EcoRI site at the 3' end). Cell lysates were immunoprecipitated by anti-FLAG monoclonal antibodies and washed immunoprecipitates were assayed for *in-vitro* autophosphorylation in the presence of calmodulin and Ca^{2+}. As shown in Fig. 2C, a single phosphorylated band of 160 kDa appeared upon fractionation of the *in-vitro* reaction products on polyacrylamide gels. This experiment provides the first direct proof that the recombinant protein has intrinsic kinase activity, as suggested by the predicted amino acid structure.

VII. Expression of DAP-1 and DAP-2 proteins in various cells and tissues

Examination of a variety of cell lines and tissues revealed that these two genes are likely to be ubiquitously expressed. Fig. 10 shows the Northern blot analysis of RNA from different hematopoietic cells probed with the DAP-1 cDNA. The 2.4 Kb mRNA transcript of this gene was detected in granulocytes (HL-60) B lymphoid (Daudi) and macrophage (U937) cells. The expression levels in the hematopoietic cells was lower than in HeLa cells. Fig. 11 shows results of examination of the mRNA expression in human embryonic tissues: brain, spleen (predominantly B-cells) and liver (predominantly erythrocytes). Again the single 2.4 Kb mRNA transcript was detected in these tissues by the DAP-1 cDNA probe.

The DAP-2 cDNA probe 2 recognized the 6.3 Kb mRNA encoded by this gene in these different tissues (Fig. 11). The embryonal liver and spleen tissues from Down syndrome seemed in this blot to express higher levels of the DAP-2 gene (compared to the GAPDH levels) while the brain tissue from Down syndrome contained higher levels of DAP-1 mRNA than the corresponding normal brain.

VIII. Cloning and sequencing of DAP-3, DAP-4 and DAP-5

Clone 259 (DAP-3) was sequenced and used to screen a K562 λgt10 cDNA library as described above for DAP-1 and DAP-2. The sequence of the (almost) full length cDNA of DAP-3 and the deduced amino acid sequence is shown in Fig. 12.

Clones 253 (DAP-4) and 260 (DAP-5) were partially sequenced as described above for DAP-1 and DAP-2, and the results are shown in Figs. 13 and 14, respectively.

IX. Identification of DAP-7

The initial microscopic observations, performed on the different HeLa cells that had been transfected with the individual rescued pTKO1 clones (described in Table 1), indicated that plasmid pKTO1-229 (group 7) conveyed similar effects to those conferred by the plasmids from group 1. It reduced the susceptibility of the cells to the IFN-γ-induced cell death but not to its cytostatic effects.

The cDNA carried by plasmid pTKO1- 229 was identified upon sequencing as a BamHI-HindIII fragment of human cathepsin D cDNA, which was present in the expression vector in the antisense orientation. The DNA probe, corresponding to fragment #229, hybridized as expected to a single endogenous 2.5 Kb mRNA, both in control and in the transfected HeLa cells. The steady state levels of cathepsin D sense mRNA were not affected by the IFN-γ treatment. In the pTKO1-229 transfected cells the DNA probe also hybridized to the composite antisense RNA. The levels of antisense cathepsin D RNA were stimulated 5-fold in response to IFN-γ

due to the presence of an ISRE enhancer element in the pTKO1 expression vector (not shown).

The cathepsin D protein was identified on immunoblots using commercially available polyclonal antibodies (Oncogene Science). It was found that in the control clones, IFN−γ prevented the appearence of the mature 34kDa chain while the 48kDa active single chain precursor was retained at abnormal high levels in these cells. It appears that this single chain precursor is the specific cathepsin form that functions during cell death.

Cathepsin D is an aspartic protease that is found normally in lysosomes where it functions in protein catabolism. Yet, in some pathological situations it has been suggested that this protease can function in the cytosol, and its activity was associated with degenerative brain changes, muscular dystrophy and connective tissue disease pathology (Matus and Green (1987); Biochemistry, 26, 8083−8036). The present invention shows for the first time that the expression of this protease is indispensable for the execution of programmed cell death that is induced by IFN−γ. Thus, cathepsin D joins the growing list of proteases that play a key role in different scenarios of programmed cell death.

The DNA sequence and amino acid sequence of cathepsin D are shown in Fig. 15 (Faust, P.L. *et al.* (1985) PNAS USA 82, 4910−4914).

CLAIMS:

1. A DNA molecule comprising a sequence selected from the group consisting of:

(a) a gene whose expression is necessary for the mediation of the cytokine-induced programmed cell death;

(b) a DNA sequence encoding the same protein or polypeptide encoded by said gene defined in (a);

(c) a modified DNA sequence of (a) or (b) in which one or more nucleic acid triplets has been added, deleted, or replaced, the protein or polypeptide encoded by the modified DNA sequence mediating the cytokine-induced programmed cell death similarly to the protein or polypeptide encoded by said gene as defined under (a) or (b);

(d) fragments of any of the DNA sequences of (a), (b) or (c), encoding a protein or a polypeptide having said biological activity;

(e) a sequence which is an antisense to the entire or part of the DNA molecule under (a) and capable of inhibiting the expression of said gene; and

(f) a modified DNA sequence of (a) or (b) in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the modified sequence having dominant negative effect manifested by the ability of said protein or polypeptide to inhibit said cytokine-induced programmed cell death.

2. A DNA molecule according to Claim 1, comprising a nucleic acid sequence selected from the group consisting of:

(a) a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 160-162 and ending at the triplet 466-468 of the sequence depicted in Fig. 6;

(b) a DNA sequence comprising a coding sequence beginning at nucleic acid triplet at position 287-289 and ending at a triplet at positions 816-818 of the sequence depicted in Fig. 6;

(c) a DNA sequence comprising a coding sequence beginning at nucleic acid triplet at position 337–339 and ending at the triplet at position 4603–4605 of the sequence depicted in Fig. 8;

(d) a DNA sequence comprising a coding sequence beginning at position 74–76 and ending at position 1268–1270 of the sequence depicted in Fig. 12;

(e) a DNA sequence comprising a sequence depicted in Fig. 13;

(f) a DNA sequence comprising a sequence depicted in Fig. 14;

(g) a DNA sequence encoding the same protein or polypeptide encoded by any one of the DNA sequences of (a) – (f);

(h) a DNA sequence as in (a) – (g) in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the sequence having essentially the same biological activity as that encoded by any one of the DNA sequences of (a) – (g), respectively;

(i) fragments of any one of the DNA sequences of (a) – (h), encoding a protein or polypeptide retaining a biological activity present in the protein or polypeptide encoded by any one of the DNA sequences under (a)–(h), respectively;

(j) a sequence which is an antisense to the entire or part of any one of the sequences under (a) – (f) or of the cathepsin D gene in Fig. 15 and capable of inhibiting the expression of said sequence; and

(k) a modified DNA sequence of any one of the sequences (a) – (f) in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the modified sequence having dominant negative effect and being capable of inhibiting the function of the protein or polypeptide encoded by any one of the sequences (a) – (f), respectively.

3. A vector comprising a DNA sequence as defined in Claim 1 or 2 and sequences required for propagating and replicating the DNA sequence in a host cell.

4. A vector according to Claim 3, being an expression vector comprising also sequences required for translation of said DNA into an mRNA.

5. A vector according to Claim 4, being an episomal plasmid constructed by using vectors pTKO1, pTKO2 and pTKO3.

6. A protein or polypeptide comprising an amino acid sequence encoded by a DNA molecule as defined in Claim 1(a)-(d) or in Claim 2(a)-([f]i).

7. A protein or polypeptide according to Claim 6, wherein one or more amino acid residues has been chemically modified.

8. A protein according to Claim 6 or 7 having the amino acid sequence as depicted in any of Figs. 6, 8, or 12.

9. Cathepsin D for use in the mediation of cytokine-induced programmed cell death.

10. An antibody directed against a protein according to any one of Claims 6-9 or against an immunogenic portion thereof.

11. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an active ingredient selected from the group consisting of:

a. a protein or polypeptide according to any one of Claims 6-9;
b. an expression vector according to either of Claims 4 or 5;
c. an antibody according to Claim 10; and
d. an inhibitor or antagonist of the protein of (a).

12. A pharmaceutical composition according to Claim 11, for inducing cytokine-induced programmed cell death, wherein said active ingredient is selected from the group consisting of:

a. a protein according to any one of Claims 6-9; and
b. an expression vector according to Claim 4 in which the DNA molecule is that of Claim 1(a)-(d) or that of Claim 2(a)-(i).

13. A pharmaceutical compositon according to claim 12 further comprising a cytokine.

14. A pharmaceutical composition according to Claim 11 for inhibiting a cytokine-induced programmed cell death, wherein said active ingredient is selected from the group consisting of:

a. an antibody of Claim 10;

b. an inhibitor or antagonist of the protein of Claims 6–9; and

c. an expression vector according to either of Claims 4 or 5, which comprises a DNA molecule of Claim 1(e) or 1(f) or Claim 2(j).

15. A method for detecting a mutation in a gene encoding a product involved in cytokine-induced programmed cell death comprising probing genomic DNA or cDNA from the individual with one or more DNA probes comprising a complete or partial sequence of the DNA sequence as defined in Claim 1 or 2.

16. A method for the screening of individuals having a predisposition to cancer, comprising:

(a) obtaining a sample of either genomic DNA from cells of the individual or cDNA produced from mRNA of said cells;

(b) adding one or more DNA probes, each of said probes comprising a complete or partial sequence of a DAP gene, or a sequence which is an antisense sequence to the complete or partial sequence of the DAP gene;

(c) providing conditions for hybridization between the DNA probe or probes and the DNA of said sample;

(d) determining on the basis of the hybridization whether the DAP gene is absent, whether there is a match between the sequence of the DNA probe or probes and a sequence in the DNA of said sample or whether there is a mismatch, a mismatch indicating a deletion or a mutation in the genomic DNA and a predisposition to cancer of the tested individual.

17. A method for screening of individuals having a predisposition to cancer by the use of antibodies which are able to distinguish between a functional protein of Claims 6 or 10 and a non-functional mutated protein.

18. A method of treatment of a disease or a disorder associated with uncontrolled, pathological cell growth, comprising administering to a

diseased individual a therapeutically effective amount of an active ingredient selected from the group consisting of:

a. a protein according to any of claims 6–9; and

b. an expression vector according to claim 4 in which the DNA molecule is that of claim 1(a)–(d) or that of claim 2(a)–(i).

19. A method of treatment of a disease according to claim 18, wherein said active ingredient is administered in conjunction with a cytokine.

20. A method of treatment of a disease or disorder associated with cytokine-induced programmed cell death, comprising administering a therapeutically effective amount of an active ingredient capable of inhibiting physiological cell death, said active ingredient being a member selected from the group consisting of:

a. an antibody of claim 10;

b. an inhibitor or antagonist of the protein of claims 6–9; and

c. an expression vector according to either of claims 4 or 5, which comprises a DNA molecule of claim 1(e), 1(f) or claim 2(j).

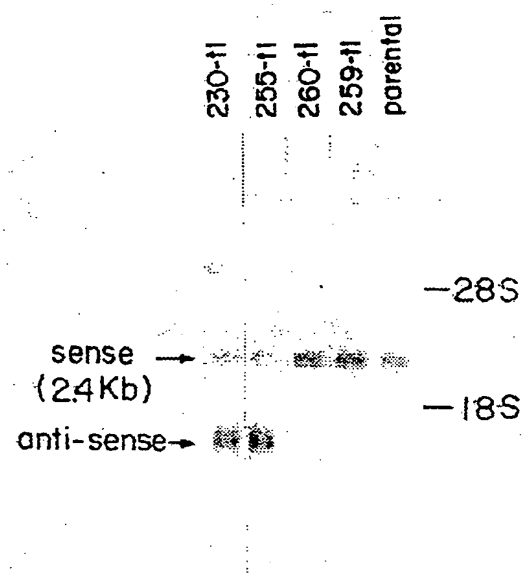

230-11
255-11
260-11
259-11
parental
—28S
sense →
(2.4Kb)
—18S
anti-sense→

FIG. 1A

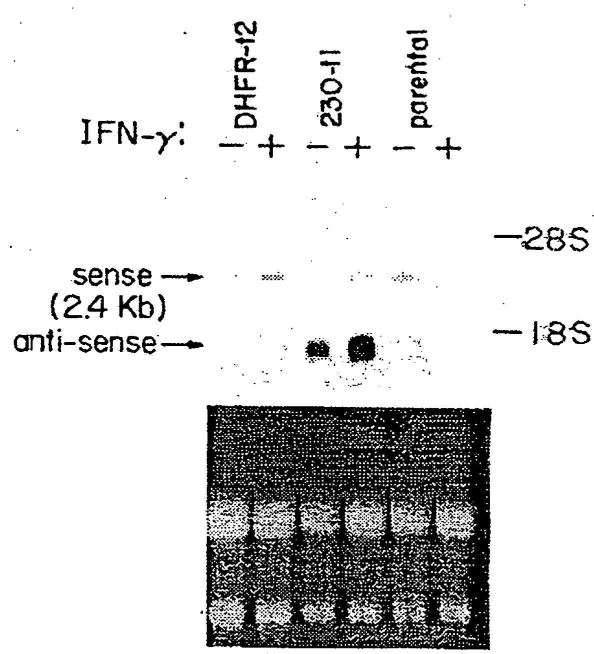

DHFR-12
230-11
parental
IFN-γ: − + − + − +
—28S
sense →
(2.4 Kb)
anti-sense→
—18S

FIG. 1B

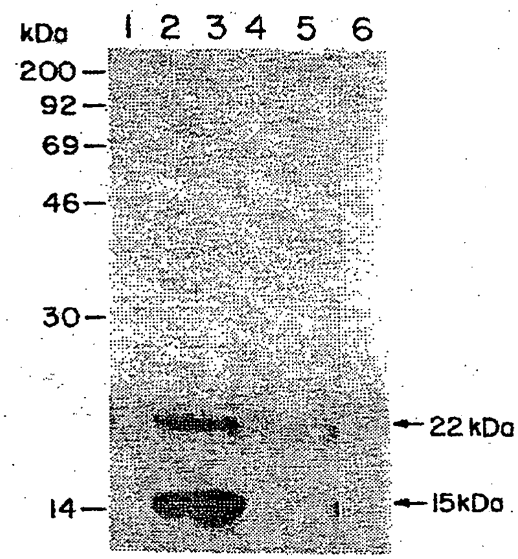

kDa
1 2 3 4 5 6
200
92
69
46
30
14
22 kDa
15kDa

FIG. 1C

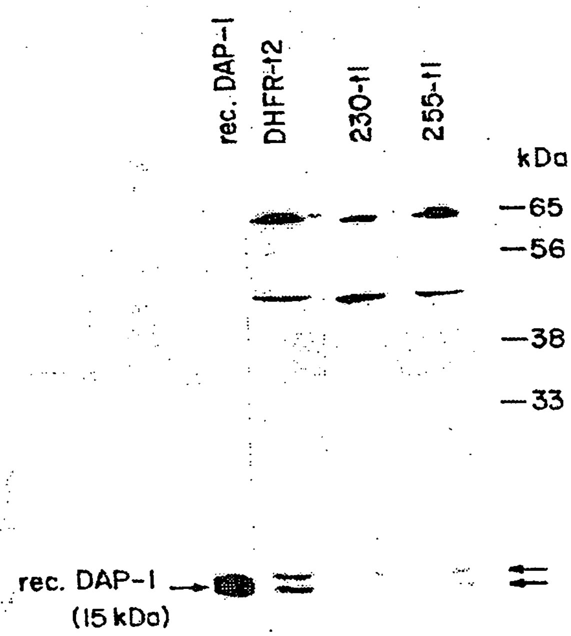

rec. DAP-1
DHFR-12
230-11
255-11
kDa
65
56
38
33
rec. DAP-1
(15 kDa)

FIG. 1D

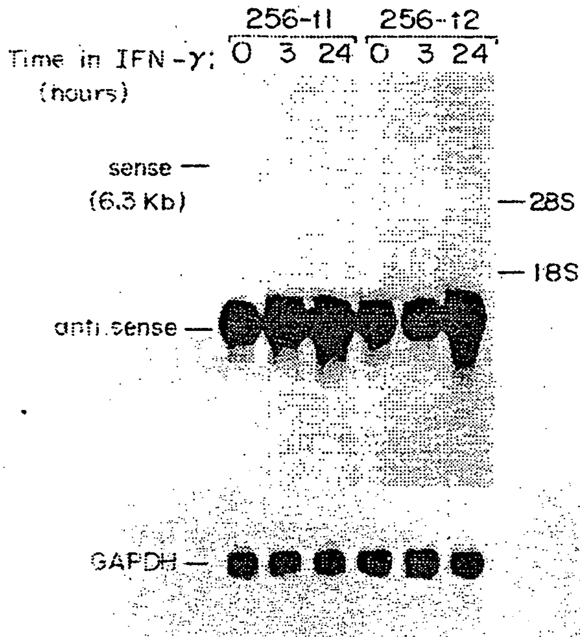


FIG. 2A

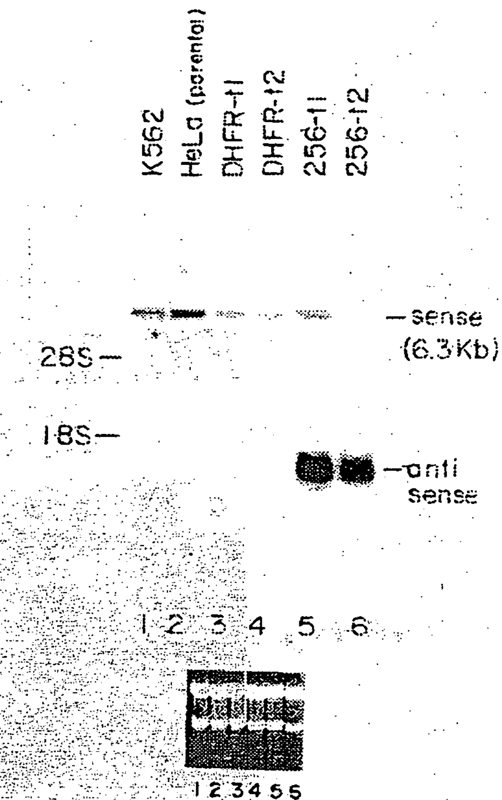


FIG. 2B

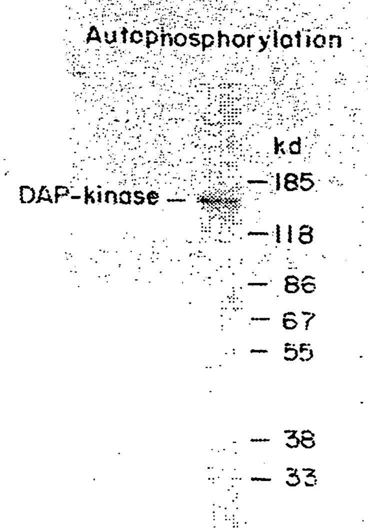


FIG. 2C

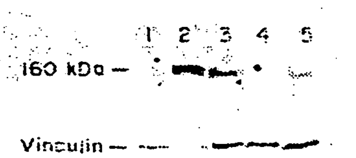


FIG. 2D

FIG. 3A(a) FIG. 3A(c)

day 3 day 8

−IFN-γ

+IFN-γ

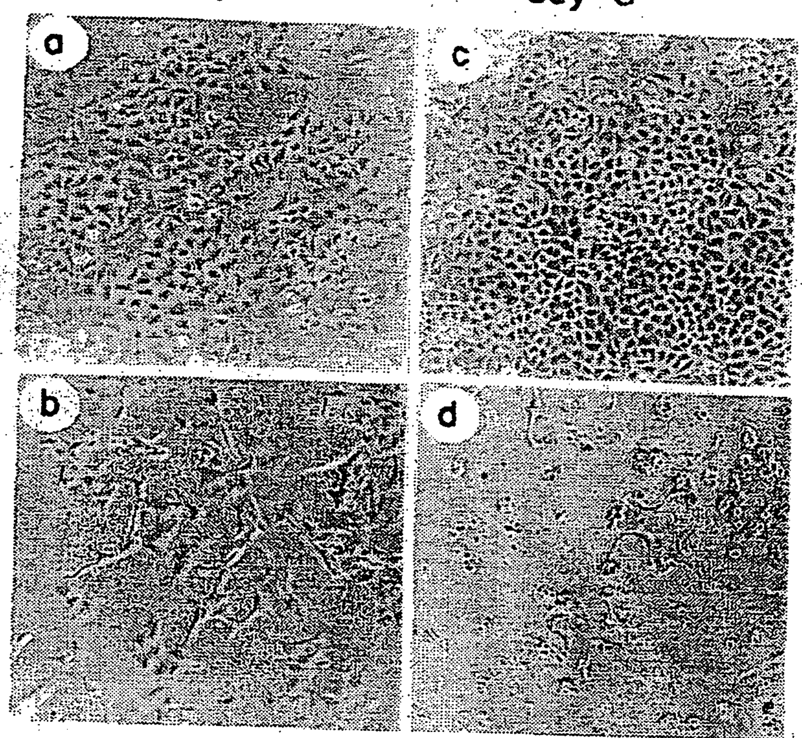


FIG. 3A(b) FIG. 3A(d)

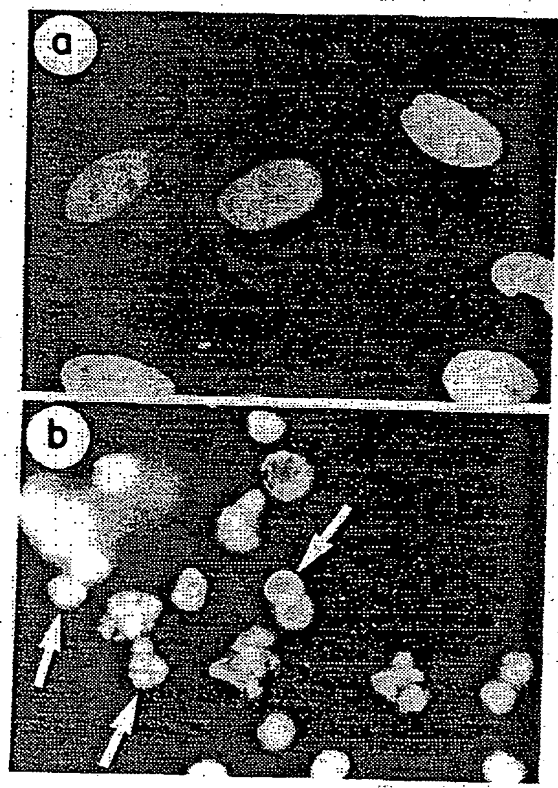


FIG. 3B(a)

FIG. 3B(b)

FIG. 3C(a) FIG. 3C(c) FIG. 3C(e)

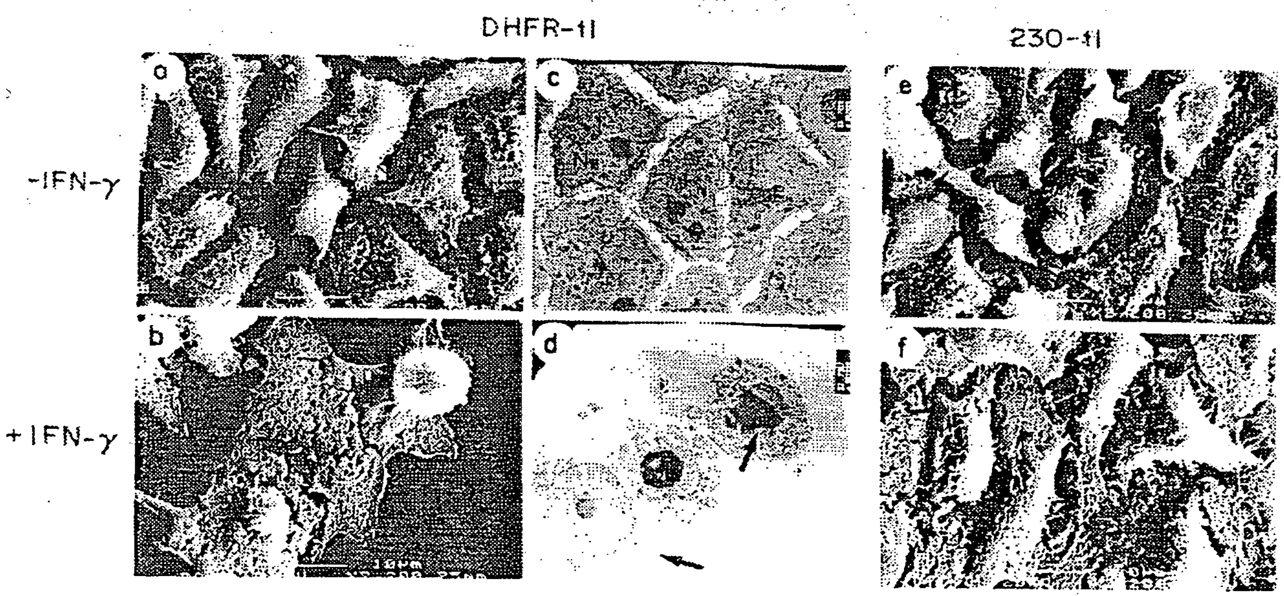
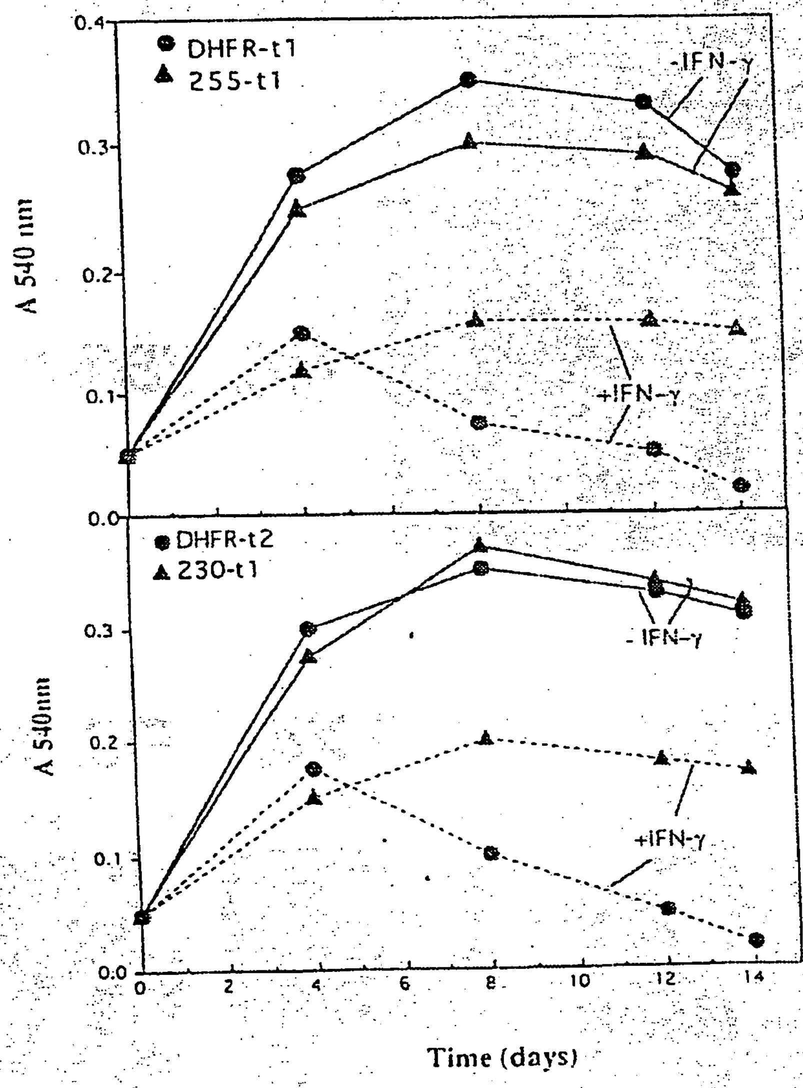
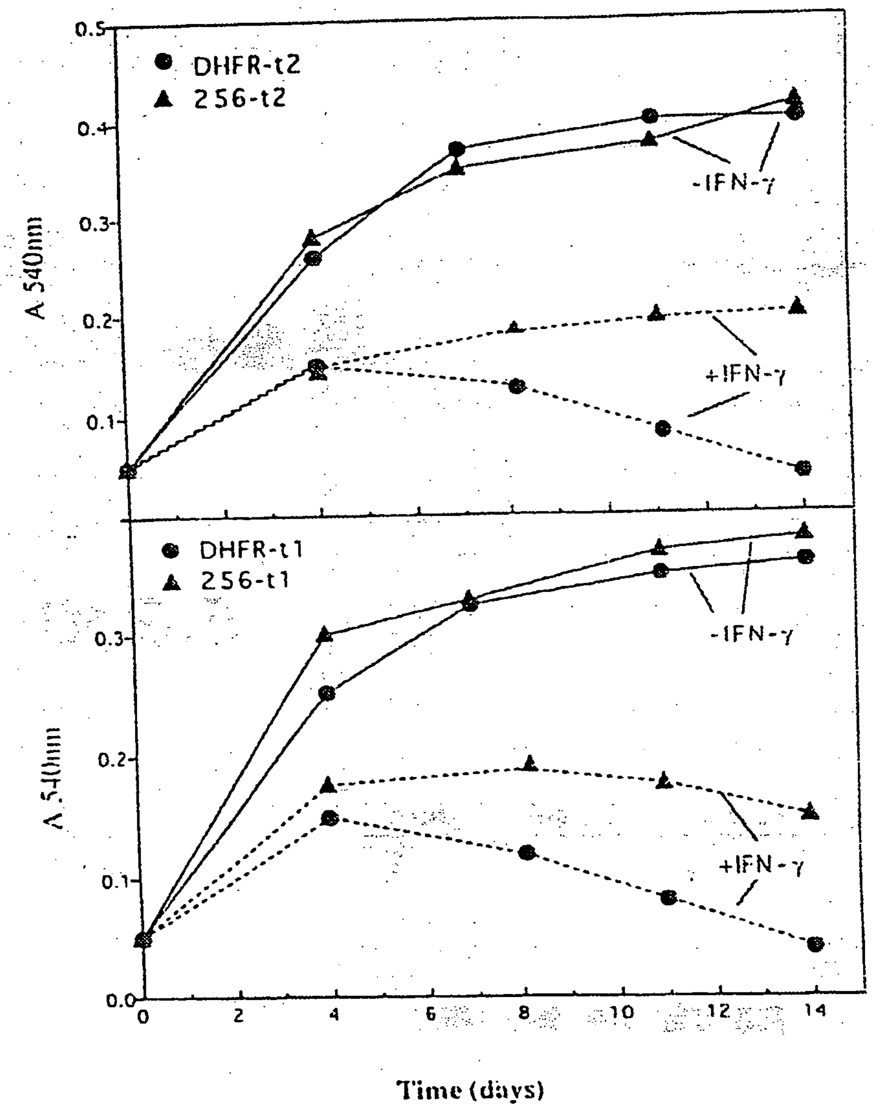


FIG. 3C(b) FIG. 3C(d) FIG. 3C(f)



DHFR-t1
255-t1
-IFN-γ
+IFN-γ
A 540 nm
0.4
0.3
0.2
0.1
0.0
DHFR-t2
230-t1
-IFN-γ
+IFN-γ
A 540nm
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
Time (days)

FIG. 4A



DHFR-t2
256-t2
-IFN-γ
+IFN-γ
A 540nm
0.5
0.4
0.3
0.2
0.1
DHFR-t1
256-t1
-IFN-γ
+IFN-γ
A 540nm
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
Time (days)

FIG. 4B

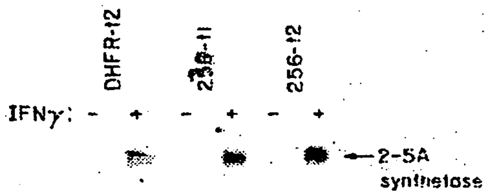


FIG. 4C

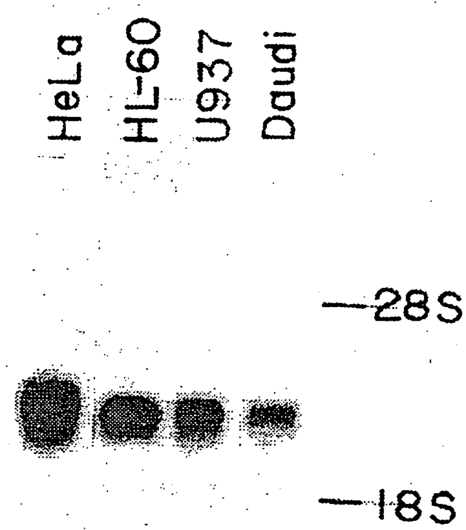


FIG. 10

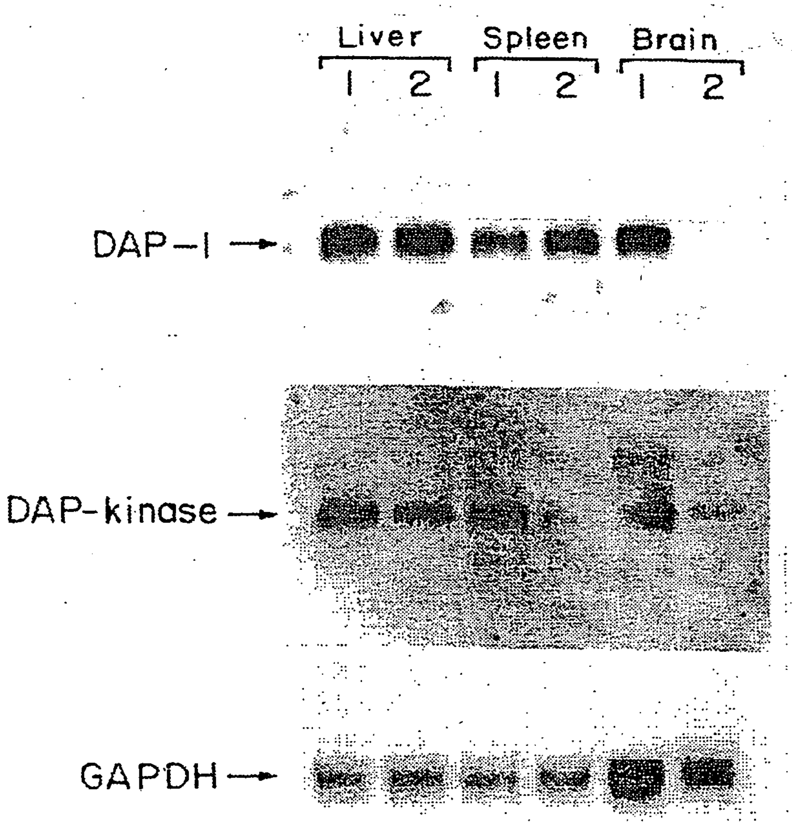


FIG. 11

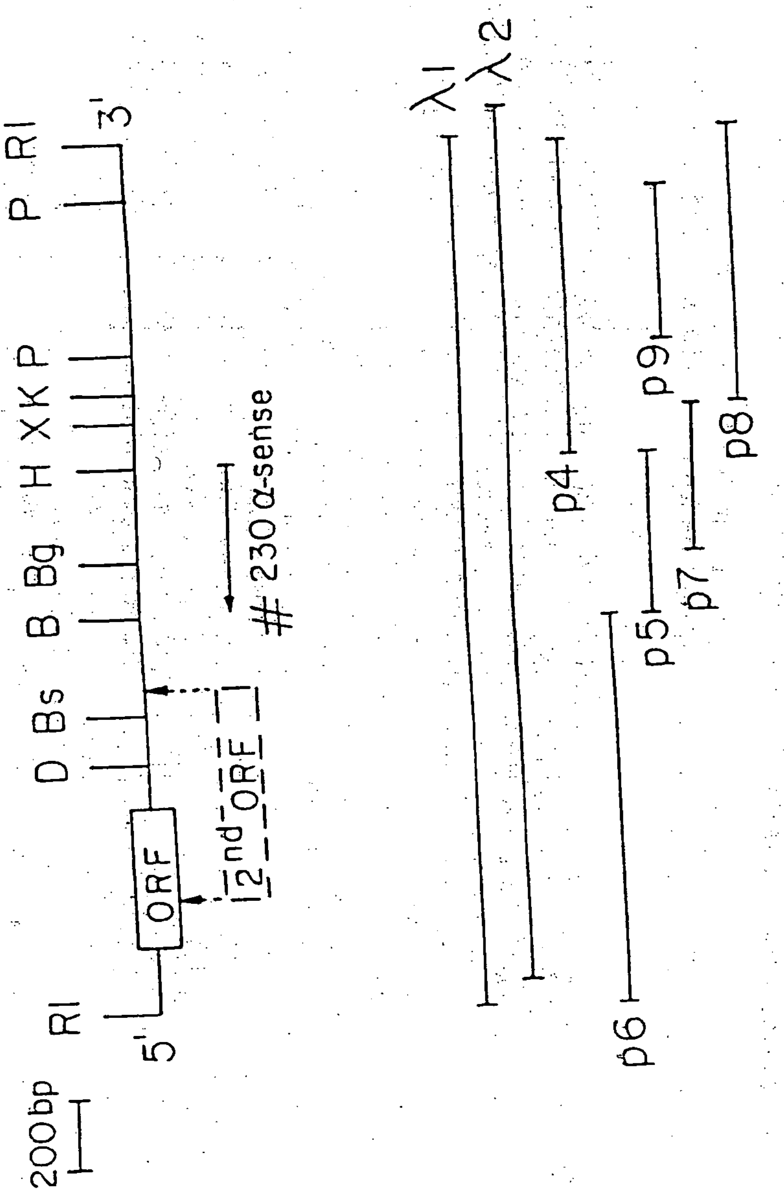

200bp
RI
5'
ORF
2nd ORF
D
Bs
B
Bg
H
X
K
P
P
RI
3'
#230 α-sense
λ1
λ2
p4
p5
p6
p7
p8
p9

FIG. 5

```
CGTGGCACTCACCCGGCTCGCGCGGCCCCGGCCGCCCACGCCGCGCGTCGTTCTCCCGCCCGCTCGCTCCCCGGCGCTCACACCTGAGCT   90
                                                                   M  S  S  P  P  E  G       7
CACTCGCGCACGCCCGCCCGGCCCGAGAACCGCGCCGCCGCCTCGGCCCCGCGGAAGCCCCGCCGCGCCATGTCTTCGCCTCCCGAAGGG  180
K  L  E  T  K  A  G  H  P  P  A  V  K  A  G  G  M  R  I  V  Q  K  H  P  H  T  G  D  T  K    37
AAACTAGAGACTAAAGCTGGACACCCGCCCGCCGTGAAAGCTGGTGGAATGCGAATTGTGCAGAAACACCCACATACAGGAGACACCAAA  270
E  E  K  D  K  D  D  Q  E  W  E  S  P  S  P  P  K  P  T  V  F  I  S  G  V  I  A  R  G  D    67
GAAGAGAAAGACAAGGATGACCAGGAATGGAAAGCCCCAGTCCACCTAAACCCACTGTGTTCATCTCTGGGGTCATCGCCCGGGGTGAC  360
K  D  F  P  P  A  A  A  Q  V  A  H  Q  K  P  H  A  S  M  D  K  H  P  S  P  R  T  Q  H  I    97
AAAGATTTCCCCCCGGCGGCTGCGCAGGTGGCTCACCAGAAGCCGCATGCCTCCATGGACAAGCATCCTTCCCCAAGAACCCAGCACATC  450
                                                                                           102
Q  Q  P  R  K  *
CAGCAGCCACGCAAGTGAGCCTGGAGTCCACCAGCCTGCCCCATGGCCCCGGCTCTGCTGCACTTGGTATTTCCCTGACAGAGAGAACCA  540
GCAGTTTCGCCCAAATCCTACTCTGCTGGGAAATCTAAGGCAAAACCAAGTGCTCTGTCCTTTGCCTTACATTTCCATATTTAAAACTAG  630
AAACAGCTTCAGCCCAAACCTTGTTTATGGGGAGTCTGGTTGGATGTCATTTGAGGATCATTGTGCCCCTAGAGGTGCCATTAGCAGAAT  720
TTGCCAAGATCCGAGAAAAATTTTAGCTTTAGTTCTATTTCAGCAGTCACCTGACGTCCTTGTCTATGGTCTTAAAAACAAGAAGGCACA  810
CATTTGAGAAGATGAGATTAAGGTTAGGAGAAAACCTCAGTCATTGCATGCTTTTTAGTATGGGCCAATAAAATCTCAACACCTGTGGGA  900
GAGTAAGAACTAAGGGAATGAGTTTGGGCGCCCCCTCATAAAGGACCTTAGAGGCAGGGAACAGCAATGCCAAATTTCCCTCTCTCGTGA  990
GATGGGGGATCCTGTGCAGGCTGATGAGGCACCCATGAGAAAAGCCGAAAAAGCATGCATCTTAGAAATAGCCCCTCAATTCCAGGAGTC 1080
AACATGCCAAAGAATGAGGCTGGAGACAGGTAGCTCCGAGGGAGGACTTCTGGCATGAGATCTCGGCACGGCAAGCCCAGCATCGCCTCA 1170
GCCCAGACAGGCTCCACCAGGAGATCAAGCAAGGGCTGCCTTTCAGGAGTCACCTCCTGAGCCACTTCAGAGTTCTGGAAGTGACCACGG 1260
ACCAGGGTGGAGGAATAGACTTCTAGTTCATTCTGGGACACTTGAGCCAGAGAGTTGAAAGCTTGGAAAGACCAGATAAGAAACCTGCCC 1350
TTTGTCTCCCTAGGGACATGAGACACCACATTCCATTTGTGCTAGAAAAACCTATCCACTGATGAGTCTAACTGTTCCAAACGCCTCCCA 1440
CCTGGTGTGCACAGCTGCCTGGGTCCATTGTCACTTGGGTGCATCAGGTTGTCCTCCGATTTTTAGATGAGTTTCCTGTCTAGAGATGTC 1530
CTAGTCTGCTCACTGGCTGGTGGCAGTAGGGTACCCTGCGTCCTCGAAAAGCCAGAGGGTTCACCTAGTCAGACGAAACTCCAGAACAGT 1620
GCTTGTGGAGGGCCTGACTGTCCTGCTCACCCACAGCCGATCTGCTGCAGGTCAGCAACTGTGTCGTGAGCAGCTGCCAACCACCAGCCT 1710
TTCTGGTGCTGTTCTCCAGTTCACGTCTGCCAGCTGGTGAGGGCAGAGGCAGACCTGGTCAGACCCAGCGCCCCTCCTCCCTGAGGGAGC 1800
ATGGCACAGCCTCACACTTGAAAGACGGTGTTTGGTTTCCCATCTAATCAACTTAAGGGAAGCCGGCATGTACCCTTCAAGGCCCTGTCA 1890
CCACCTATTTTCCTGATCAGTTGGTATAAACTGAGGGTGGCTTTTAGAGACCCAGACTTGGTTGGCAGCGCTGCCATGGAACACCCCAGC 1980
AAGCACCTCCCAGCCTGCCTTTCGGAGCAGCACCCAGGAGGGGATGCCGCGCTCCAGCAACACCAGGTCAGGCCTGTGCAGACCCCTGCC 2070
CTGCCGCTGCAGAAATCCAGAAGCATCCTTAATGCTTCTCAGTCTTCAGCCAGAGGGAGGGCTGTTATTTCCAGAGGTGCGCTTTTTATG 2160
TACTTTTAGCTAGATGTGGCATGCATCTGTGAGCTTTAGATCATTAAATCCAAAATGTTTGCCTAAATGAGG                   2232
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FIG. 6

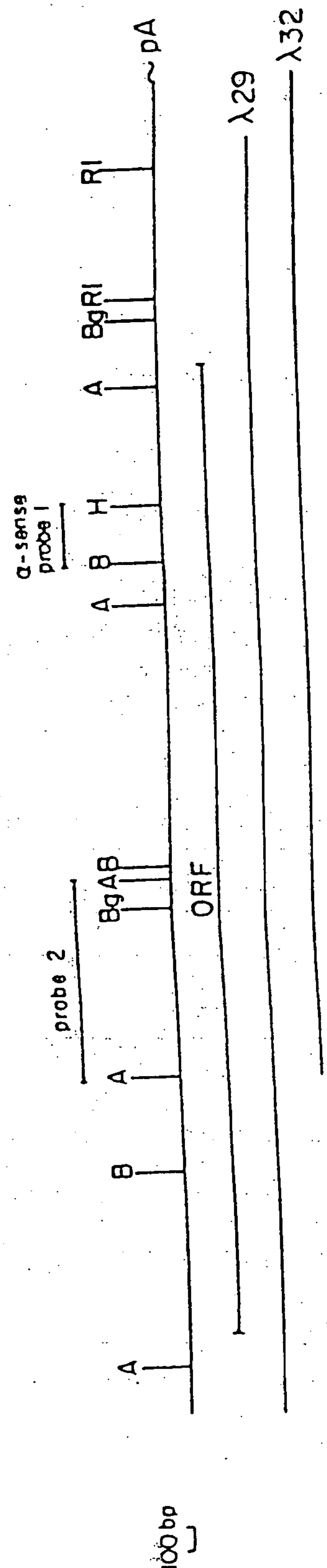
~pA
RI
BgRI
A
H
B
A
a-sense
probe 1
BgAB
ORF
probe 2
A
B
A
λ29
λ32
100bp

FIG. 7

CGGAGGACAGCCGGACCGAGCCAACGCCGGGGACTTTGTTCCCTCCACGGAGGGGACTCGGCAACTCGCAGCGGCAGGGTCTGGGGCCGG 90
CGCCTGGGAGGGATCTGCGCCCCCCACTCACTCCCTAGCTGTGTTCCCGCCGCCGCCCCGGCTAGTCTCCGGCGCTGGCGCCTATGGTCG 180
GCCTCCGACAGCGCTCCGGAGGGACCGGGGGAGCTCCCAGGCGCCCGGGACTGGAGACTGATGCATGAGGGGCCTACGGAGGCGCAGGAG 270

M T V F R Q E N 8
CGGTGGTGATGGTCTGGGAAGCGGAGCTGAAGTCCCCTGGGCTTTGGTGAGGCGTGACAGTTTATC$\boxed{\text{ATG}}$ACCGTGTTCAGGCAGGAAAAC 360
PROTEIN KINASE DOMAIN

V D D Y Y D T G E E L G S G Q F A V V K K C R E K S T G L Q 38
GTGGATGATTACTACGACACCGGCGAGGAACTTGGCAGTGGACAGTTTGCGGTTGTGAAGAAATGCCGTGAGAAAAGTACCGGCCTCCAG 450

Y P A K F I K K R R T K S S R R G V S R E D I E R E V S I L 68
TATCCCGCCAAATTCATCAAGAAAAGGAGGACTAAGTCCAGCCGGCGGGGTGTGAGCCGCGAGGACATCGAGCGGGAGGTCAGCATCCTG 540

K E I Q H P N V I T L H E V Y E N K T D V I L I L E L V A G 98
AAGGAGATCCAGCACCCCAATGTCATCACCCTGCACGAGGTCTATGAGAACAAGACGGACGTCATCCTGATCTTGGAACTCGTTGCAGGT 630

G E L F D F L A E K E S L T E E E A T E F L K Q I L N G V Y 128
GGCGAGCTGTTTGACTTCTTAGCTGAAAAGGAATCTTTAACTGAAGAGGAAGCAACTGAATTTCTCAAACAAATTCTTAATGGTGTTTAC 720

Y L H S L Q I A H F D L K P E N I M L L D R N V P K P R I K 158
TACCTGCACTCCCTTCAAATCGCCCACTTTGATCTTAAGCCTGAGAACATAATGCTTTTGGATAGAAATGTCCCCAAACCTCGGATCAAG 810

I I D F G N E F K N I F G T P E F V A P E I V N Y E P L G L 188
ATCATTGACTTTGGAAATGAATTTAAAAACATATTTGGGACTCCAGAGTTTGTCGCTCCTGAGATAGTCAACTATGAACCTCTTGGTCTT 900

E A D M W S I G V I T Y I L L S G A S P F L G D T K Q E T L 218
GAGGCAGATATGTGGAGTATCGGGGTAATAACCTATATCCTCCTAAGTGGGGCCTCCCCATTTCTTGGAGACACTAAGCAAGAAACGTTA 990

A N V S A V N Y E F E D E Y F S N T S A L A K D F I R R L L 248
GCAAATGTATCCGCTGTCAACTACGAATTTGAGGATGAATACTTCAGTAATACCAGTGCCCTAGCCAAAGATTTCATAAGAAGACTTCTG 1080

PROTEIN KINASE DOMAIN

V K D P K K R M T I Q D S L Q H P W I K P K D T Q Q A L S R 278
GTCAAGGATCCAAAGAAGAGAATGACAATTCAAGATAGTTTGCAGCATCCCTGGATCAAGCCTAAAGATACACAACAGGCACTTAGTAGA 1170

calmodulin regulatory region

K A S A V N M E K F K K F A A R K K W K Q S V R L I S L C Q 308
AAAGCATCAGCAGTAAACATGGAGAAATTCAAGAAGTTTGCAGCCCGGAAAAAATGGAAACAATCCGTTCGCTTGATATCACTGTGCCAA 1260

R L S R S F L S R S N M S V A R S D D T L D E E D S F V M K 338
AGATTATCCAGGTCATTCCTGTCCAGAAGTAACATGAGTGTTGCCAGAAGCGATGATACTCTGGATGAGGAAGACTCCTTTGTGATGAAA 1350

A I I H A I N D D N V P G L Q H L L G S L S N Y D V N Q P N 368
GCCATCATCCATGCCATCAACGATGACAATGTCCCAGGCCTGCAGCACCTTCTGGGCTCATTATCCAACTATGATGTTAACCAACCCAAC 1440
or1

K H G T P P L L I A A G C G N I Q I L Q L L I K R G S R I D 398
AAGCACGGGACACCTCCATTACTCATTGCTGCTGGCTGTGGGAATATTCAAATACTACAGTTGCTCATTAAAAGAGGCTCGAGAATCGAT 1530
or2

V Q D K G G S N A V Y W A A R H G H V D T L K F L S E N K C 428
GTCCAGGATAAGGGCGGGTCCAATGCCGTCTACTGGGCTGCTCGGCATGGCCACGTCGATACCTTGAAATTTCTCAGTGAGAACAAATGC 1620
or3

P L D V K D K S G E M A L H V A A R Y G H A D V A Q V T C A 458
CCTTTGGATGTGAAAGACAAGTCTGGAGAGATGGCCCTCCACGTGGCAGCTCGCTATGGCCATGCTGACGTGGCTCAAGTTACTTGTGCA 1710
or4

A S A Q I P I S R T K E E E T P L H C A A W H G Y Y S V A K 488
GCTTCGGCTCAAATCCCAATATCCAGGACAAAGGAAGAAGAAACCCCCCTGCACTGTGCTGCTTGGCACGGCTATTACTCTGTGGCCAAA 1800
or5

A L C E A G C N V N I K N R E G E T P L L T A S A R G Y H D 518
GCCCTTTGTGAAGCCGGCTGTAACGTGAACATCAAGAACCGAGAAGGAGAGACGCCCCTCCTGACAGCCTCTGCCAGGGGCTACCACGAC 1890
or6

I V E C L A E H G A D L N A C D K D G H I A L H L A V R R C 548
ATCGTGGAGTGTCTGGCCGAACATGGAGCCGACCTTAATGCTTGCGACAAGGACGGACACATTGCCCTTCATCTGGCTGTAAGACGGTGT 1980
or7

Q M E V I K T L L S Q G C F V D Y Q D R H G N T P L H V A C 578
CAGATGGAGGTAATCAAGACTCTCCTCAGCCAAGGGTGTTTCGTCGATTATCAAGACAGGCACGGCAATACTCCCCTCCATGTGGCATGT 2070

K D G N M P I V V A L C E A N C N L D I S N K Y G R T P L H 608
AAAGATGGCAACATGCCTATCGTGGTGGCCCTCTGTGAAGCAAACTGCAATTTGGACATCTCCAACAAGTATGGGCGAACGCCTCTGCAC 2160
or8

P-loop 1

L A A N N G I L D V V R Y L C L M G A S V E A L T T D G K T 638
CTTGCGGCCAACAACGGAATCCTAGACGTGGTCCGGTATCTCTGTCTGATGGGAGCCAGCGTTGAGGCGCTGACCACGGACGGAAAGACG 2250

A E D L A R S E Q H E H V A G L L A R L R K D T H R G L F I 668
GCAGAAGATCTTGCTAGATCGGAACAGCACGAGCACGTAGCAGGTCTCCTTGCAAGACTTCGAAAGGATACGCACCGAGGACTCTTCATC 2340

P-loop 2

Q Q L R P T Q N L Q P R I K L K L F G H S G S G K T T L V E 698
CAGCAGCTCCGACCCACACAGAACCTGCAGCCAAGAATTAAGCTCAAGCTGTTTGGCCACTCGGGATCCGGGAAAACCACCCTTGTAGAA 2430

S L K C G L L R S F F R R R R P R L S S T N S S R F P P S P 728
TCTCTCAAGTGTGGGCTGCTGAGGAGCTTTTTCAGAAGGCGTCGGCCCAGACTGTCTTCCACCAACTCCAGCAGGTTCCCACCTTCACCC 2520

L A S K P T V S V S I N N L Y P G C E N V S V R S R S M M F 758
CTGGCTTCTAAGCCCACAGTCTCAGTGAGCATCAACAACCTGTACCCAGGCTGCGAGAACGTGAGTGTGAGGAGCCGCAGCATGATGTTC 2610

FIG. 8

E P G L T K G H L E V F V A P T H H P H C S A D D Q S T K A 788
GAGCCGGGTCTTACCAAAGGGATGCTGGAGGTGTTTGTGGCCCCGACCCACCACCCGCACTGCTCGGCCGATGACCAGTCCACCAAGGCC 2700

I D I Q N A Y L N G V G D F S V H E F S G N P V Y F C C Y D 818
ATCGACATCCAGAACGCTTATTTGAATGGAGTTGGCGATTTCAGCGTGTGGGAGTTCTCTGGAAATCCTGTGTATTTCTGCTGTTATGAC 2790

Y F A A N D P T S I H V V V F S L E E P Y E I Q L N P V I F 848
TATTTTGCTGCAAATGATCCCACGTCAATCCATGTTGTTGTCTTTAGTCTAGAAGAGCCCTATGAGATCCAGCTGAACCCAGTGATTTTC 2880

H L S F L K S L V P V E E P I A F G G K L K N P L Q V V L V 878
TGGCTCAGTTTCCTGAAGTCCCTTGTCCCAGTTGAAGAACCCATAGCCTTCGGTGGCAAGCTGAAGAACCCACTCCAAGTTGTCCTGGTG 2970

A T H A D I M N V P R P A G G E F G Y D K D T S L L K E I R 908
GCCACCCACGCTGACATCATGAATGTTCCTCGACCGGCTGGAGGCGAGTTTGGATATGACAAAGACACATCGTTGCTGAAAGAGATTAGG 3060

N R F G N D L H I S N K L F V L D A G A S G S K D H K V L R 938
AACAGGTTTGGAAATGATCTTCACATTTCAAATAAGCTGTTTGTTCTGGATGCTGGGGCTTCTGGGTCAAAGGACATGAAGGTACTTCGA 3150

N H L Q E I R S Q I V S V C P P H T H L C E K I I S T L P S 968
AATCATCTGCAAGAAATACGAAGCCAGATTGTTTCGGTCTGTCCTCCCATGACTCACCTGTGTGAGAAAATCATCTCCACGCTGCCTTCC 3240

W R K L N G P N Q L M S L Q Q F V Y D V Q D Q L N P L A S E 998
TGGAGGAAGCTCAATGGACCCAACCAGCTGATGTCGCTGCAGCAGTTTGTGTACGACGTGCAGGACCAGCTGAACCCCCTGGCCAGCGAG 3330

E D L R R I A Q Q L H S T G E I N I M Q S E T V Q D V L L L 1028
GAGGACCTCAGGCGCATTGCTCAGCAGCTCCACAGCACAGGCGAGATCAACATCATGCAAAGTGAAACAGTTCAGGACGTGCTGCTCCTG 3420

D P R W L C T N V L G K L L S V E T P R A L H H Y R G R Y T 1058
GACCCCCGCTGGCTCTGCACAAACGTCCTGGGGAAGTTGCTGTCCGTGGAGACCCCACGGGCGCTGCACCACTACCGGGGCCGCTACACC 3510

V E D I Q R L V P D S D V E E L L Q I L D A M D I C A R D L 1088
GTGGAGGACATCCAGCGCCTGGTGCCCGACAGCGACGTGGAGGAGCTGCTGCAGATCCTCGATGCCATGGACATCTGCGCCCGGGACCTG 3600

S S G T M V D V P A L I K T D N L H R S W A D E E D E V M V 1118
AGCAGCGGGACCATGGTGGACGTCCCAGCCCTGATCAAGACAGACAACCTGCACCGCTCCTGGGCTGATGAGGAGGACGAGGTGATGGTG 3690

Y G G V R I V P V E H L T P F P C G I F H K V Q V N L C R W 1148
TATGGTGGCGTGCGCATCGTGCCCGTGGAACACCTCACCCCCTTCCCATGTGGCATCTTTCACAAGGTCCAGGTGAACCTGTGCCGGTGG 3780

I H Q Q S T E G D A D I R L W V N G C K L A N R G A E L L V 1178
ATCCACCAGCAAAGCACAGAGGGCGACGCGGACATCCGCCTGTGGGTGAATGGCTGCAAGCTGGCCAACCGTGGGGCCGAGCTGCTGGTG 3870

L L V N H G Q G I E V Q V R G L E T E K I K C C L L L D S V 1208
CTGCTGGTCAACCACGGCCAGGGCATTGAGGTCCAGGTCCGTGGCCTGGAGACGGAGAAGATCAAGTGCTGCCTGCTGCTGGACTCGGTG 3960

C S T I E N V M A T T L P G L L T V K H Y L S P Q Q L R E H 1238
TGCAGCACCATTGAGAACGTCATGGCCACCACGCTGCCAGGGCTCCTGACCGTGAAGCATTACCTGAGCCCCCAGCAGCTGCGGGAGCAC 4050

H E P V M I Y Q P R D F F R A Q T L K E T S L T N T M G G Y 1268
CATGAGCCCGTCATGATCTACCAGCCACGGGACTTCTTCCGGGCACAGACTCTGAAGGAAACCTCACTGACCAACACCATGGGGGGGTAC 4140

K E S F S S I M C F G C H D V Y S Q A S L G M D I H A S D L 1298
AAGGAAAGCTTCAGCAGCATCATGTGCTTCGGGTGTCACGACGTCTACTCACAGGCCAGCCTCGGCATGGACATCCATGCATCAGACCTG 4230

N L L T R R K L S R L L D P P D P L G K D W C L L A M N L G 1328
AACCTCCTCACTCGGAGGAAACTGAGTCGCCTGCTGGACCCGCCCGACCCCCTGGGGAAGGACTGGTGCCTTCTCGCCATGAACTTAGGC 4320

L P D L V A K Y N T N N G A P K D F L P S P L H A L L R E W 1358
CTCCCTGACCTCGTGGCAAAGTACAACACCAATAACGGGGCTCCCAAGGATTTCCTCCCCAGCCCCCTCCACGCCCTGCTGCGGGAATGG 4410

T T Y P E S T V G T L M S K L R E L G R R D A A D L L L K A 1388
ACCACCTACCCTGAGAGCACAGTGGGCACCCTCATGTCCAAACTGAGGGAGCTGGGTCGCCGGGATGCCGCAGACCTTTTGCTGAAGGCA 4500

S S V F K I N L D G N G Q E A Y A S S C N S G T S Y N S I S 1418
TCCTCTGTGTTCAAAATCAACCTGGATGGCAATGGCCAGGAGGCCTATGCCTCGAGCTGCAACAGCGGCACCTCTTACAATTCCATTAGC 4590

S V V S R 1423
TCTGTTGTATCCCGG$\boxed{TGA}$GGGCAGCCTCTGGCTTGGaCAGGGTCTGTTTGGACTGCAGAACCAAGGGGGTGATGTAGCCCATCCTTCCCT 4680
TTGGAGATGCTGAGGGTGTTTCTTCCTGCACCCACAGCCAGGGGGATGCCACTCCTCCCTCCGGCTTGACCTGTTTCTCTGCCGCTACCT 4770
CCCTCCCCGTCTCATTCCGTTGTCTGTGGATGGTCATTGCAGTTTAAGAGCAGAACAGATCTTTTACTTTGGCCGCTTGAAAAGCTAGTG 4860
TACCTCCTCTCAGTGTTTTGGACTCCATCTCTCATCCTCCAGTACCTTGCTTCTTACTGATAATTTTGCTGGAATTCCTAACTTTTCAAT 5950
GACATTTTTTTTAACTATCATATTGATTGTCCTTTAAAAAAGAAAAGTGCAT<u>ATTTA</u>TCCAAAATGTGTATTTCTTATACGCTTTTCTGT 5040
GTTATACCATTTCCTCAGCTTATCTCTTTTATATTTGTAGGAGAAACTCCCATGTATGGAATCCCACTGTATGA<u>TTTAT</u>AAACAGACAAT 5130
ATGTGAGTGCCTTTTGCAGAAGAGGGTGTGTTTGAAATCATCGGAGTCAGCCAGGAGCTGTCACCAAGGAAACGCTACCTCTCTGTCCCT 5220
TGCTGTATGCTGATCATCGCCAGAGGTGCTTCAGCCTGAGTTTTGTTTTGTATTGTTTTCTGACAGTTTTTCTGTTTTGTTTGGCAAGGA 5310
AAGGGGAGAAGGGAATCCTCCTCCAGGGTGATTTTATGATCAGTGTTGTTGCTCTAGGAAGACATTTTTCCGTTTGCTTTTGTTCCAATG 5400
TCAATGTGAACGTCCACATGAAACCTACACACTGTCATGCTTCATCATTCCCTCTCATCTCAGGTAGAAGGTTGACACAGTTGTAGGGTT 5490
ACAGAGACCTATGTAAGAATTCAGAAGACCCCTGACTCATCATTTGTGGCAGTCCCTTATAATTGGTGCATAGCAGATGGTTTCCACATT 5580
TAGATCCTGGTTTCATAACTTCCTGTACTTGAAGTCTAAAAGCAGAA<u>AATAAA</u>GGAAGCAAGTTTTCTTCCATGATTTTAAATTGTGATC 5670
GAGTTTTAAATTGATAGGAGGGAACATGTCCTAATTCTTCTGTCCTGAGAAGCATGTAATGTTAATGTTATATCATATGTATATATATAT 5760
ATGCACTATGTATATACATATATATTAATACTGGTATTTTTACTTAATCTATAAAATGTCGTTAAAAAGTTGTTTGTTTTTTTCTTTTTT 5850
TATA<u>AATAAA</u>CTGTTGCTCGTTAAAAAAAAAAAAAA 5866

FIG. 8 (cont.)

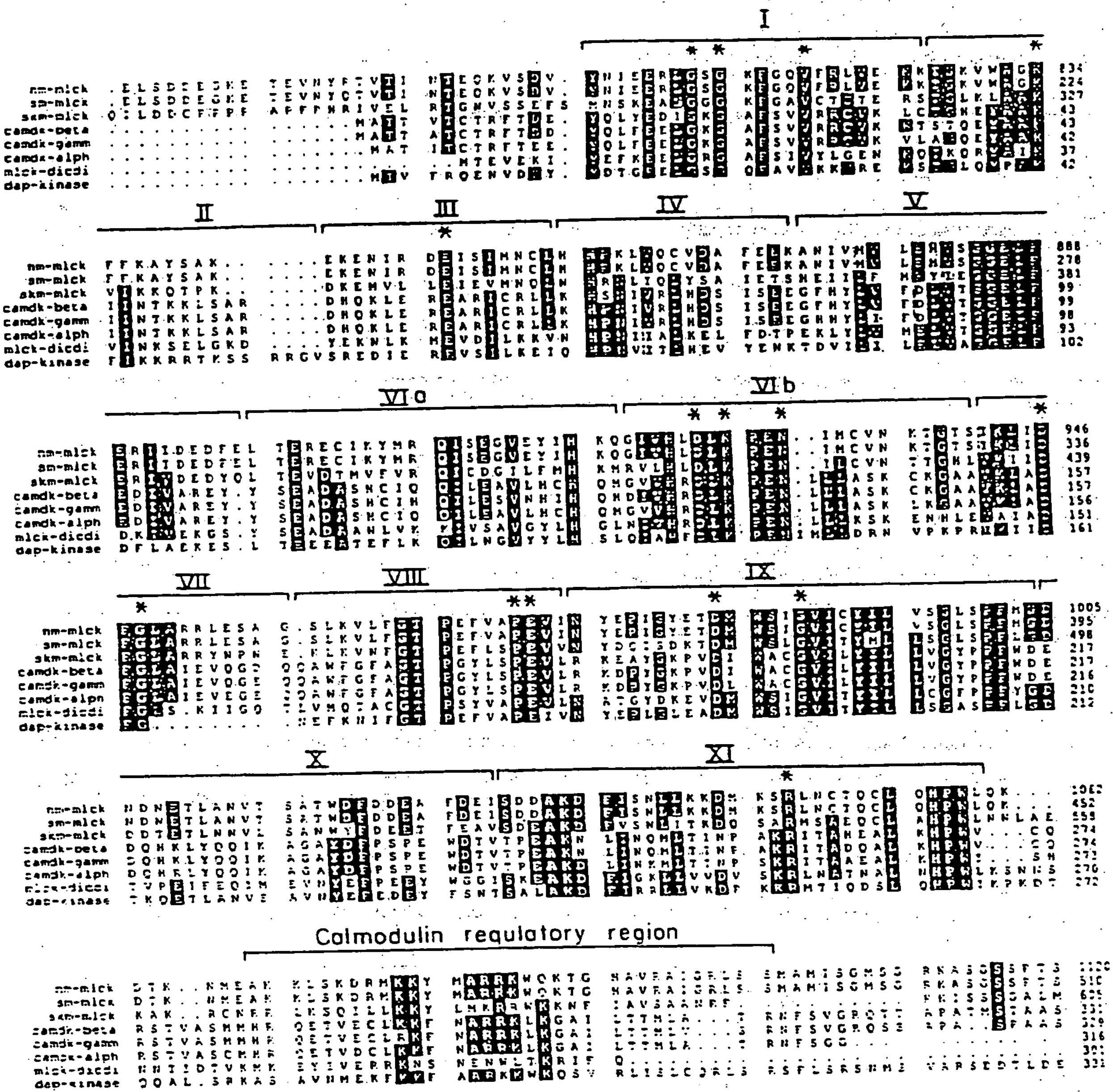
I
II
III
IV
V
VIa
VIb
VII
VIII
IX
X
XI
Calmodulin regulatory region
nm-mlck
sm-mlck
skm-mlck
camdk-beta
camdk-gamm
camdk-alph
mlck-dicdi
dap-kinase

FIG. 9A

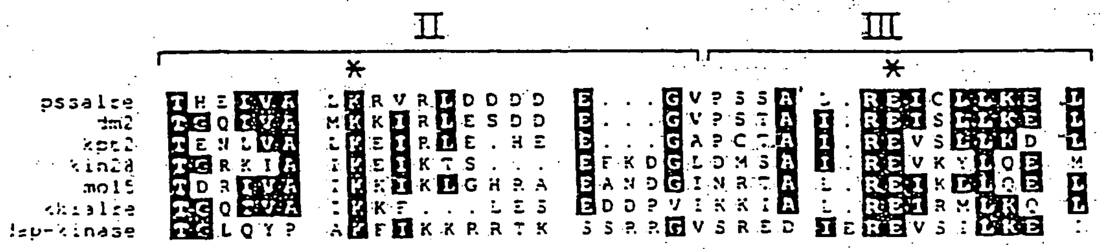

FIG. 9B

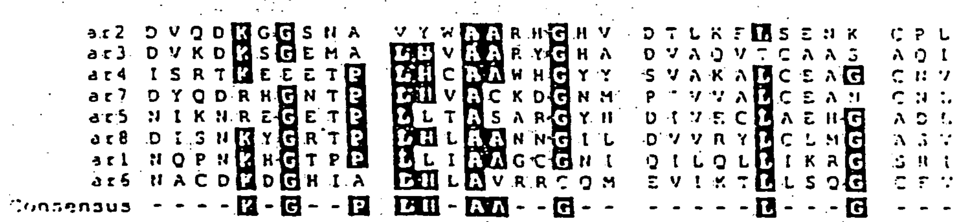

FIG. 9C

```
   1  GAATTCCGCCGGCCCCAGGCAGCGTGTGTCGGTCGCCTAGGCTGGAGAACTAGTCCTCGA   60
  61  CTCACGTGCAAGGATGATGCTGAAAGGAATAACAAGGCTTATCTCTAGGATCCATAAGTT  120
                    M  M  L  K  G  I  T  R  L  I  S  R  I  H  K  L
 121  GGACCCTGGGCGTTTTTACACATGGGGACCCAGGCTCGCCAAAGCATTGCTGCTCACCT  180
       D  P  G  R  F  L  H  M  G  T  Q  A  R  Q  S  I  A  A  H  L
 181  AGATAACCAGGTTCCAGTTGAGAGTCCGAGAGCTATTTCCCGCACCAATGAGAATGACCC  240
       D  N  Q  V  P  V  E  S  P  R  A  I  S  R  T  N  E  N  D  P
 241  GGCCAAGCATGGGGATCAGCACGAGGGTCAGCACTACAACATCTCCCCCCAGGATTTGGA  300
       A  K  H  G  D  Q  H  E  G  Q  H  Y  N  I  S  P  Q  D  L  E
 301  GACTGTATTTCCCCATGGCCTTCCTCCTCGCTTTGTGATGCAGGTGAAGACATTCAGTGA  360
       T  V  F  P  H  G  L  P  P  R  F  V  M  Q  V  K  T  F  S  E
 361  AGCTTGCCTGATGGTAAGGAAACCAGCCCTAGAACTTCTGCATTACCTGAAAAACACCAG  420
       A  C  L  M  V  R  K  P  A  L  E  L  L  H  Y  L  K  N  T  S
 421  TTTTGCTTATCCAGCTATACGATATCTTCTGTATGGAGAGAAGGGAACAGGAAAAACCCT  480
       F  A  Y  P  A  I  R  Y  L  L  Y  G  E  K  G  T  G  K  T  L
 481  AAGTCTTTGCCATGTTATTCATTTCTGTGCAAAACAGGACTGGCTGATACTACATATTCC  540
       S  L  C  H  V  I  H  F  C  A  K  Q  D  W  L  I  L  H  I  P
 541  AGATGCTCATCTTTGGGTGAAAAATTGTCGGGATCTTCTGCAGTCCAGCTACAACAAACA  600
       D  A  H  L  W  V  K  N  C  R  D  L  L  Q  S  S  Y  N  K  Q
 601  GCGCTTTGATCAACCTTTAGAGGCTTCAACCTGGCTGAAGAATTTCAAAACTACAAATGA  660
       R  F  D  Q  P  L  E  A  S  T  W  L  K  N  F  K  T  T  N  E
 661  GCGCTTCCTGAACCAGATAAAAGTTCAAGAGAAGTATGTCTGGAATAAGAGAGAAAGCAC  720
       R  F  L  N  Q  I  K  V  Q  E  K  Y  V  W  N  K  R  E  S  T
 721  TGAGAAAGGGAGTCCTCTGGGAGAAGTGGTTGAACAGGGCATAACACGGGTGAGGAACGC  780
       E  K  G  S  P  L  G  E  V  V  E  Q  G  I  T  R  V  R  N  A
 781  CACAGATGCAGTTGGAATTGTGCTGAAAGAGCTAAAGAGGCAAAGTTCTTTGGGTATGTT  840
       T  D  A  V  G  I  V  L  K  E  L  K  R  Q  S  S  L  G  M  F
 841  TCACCTCCTAGTGGCCGTGGATGGAATCAATGCTCTTTGGGGAAGAACCACTCTGAAAAG  900
       H  L  L  V  A  V  D  G  I  N  A  L  W  G  R  T  T  L  K  R
 901  AGAAGATAAAAGCCCGATTGCCCCCGAGGAATTAGCACTTGTTCACAACTTGAGGAAAAT  960
       E  D  K  S  P  I  A  P  E  E  L  A  L  V  H  N  L  R  K  M
 961  GATGAAAAATGATTGGCATGGAGGCGCCATTGTGTCGGCTTTGAGCCAGACTGGGTCTCT 1020
       M  K  N  D  W  H  G  G  A  I  V  S  A  L  S  Q  T  G  S  L
1021  CTTTAAGCCCCGGAAAGCCTATCTGCCCCAGGAGTTGCTGGGAAAGGAAGGATTTGATGC 1080
       F  K  P  R  K  A  Y  L  P  Q  E  L  L  G  K  E  G  F  D  A
1081  CCTGGATCCCTTTATTCCCATCCTGGTTTCCAACTATAACCCAAAGGAATTTGAAAGTTG 1140
       L  D  P  F  I  P  I  L  V  S  N  Y  N  P  K  E  F  E  S  C
1141  TATTCAGTATTATTTGGAAAACAATTGGCTTCAACATGAGAAAGCTCCTACAGAAGAAGG 1200
       I  Q  Y  Y  L  E  N  N  W  L  Q  H  E  K  A  P  T  E  E  G
1201  GAAAAAAGAGCTGCTGTTCCTAAGTAACGCGAACCCCTCGCTGCTGGAGCGGCACTGTGC 1260
       K  K  E  L  L  F  L  S  N  A  N  P  S  L  L  E  R  H  C  A
1261  CTACCTCTAAGCCAAGATCACAGCATGTGAGGAAGACAGTGGACATCTGCTTTATGCTGG 1320
       Y  L
1321  ACCCAGTAAGATGAGGAAGTCGGGCAGTACACAGGAAGAGGAGCCAGGCCCTTGTACCTA 1380
1381  TGGGATTGGACAGGACTGCAGTTGGCTCTGGACCTGCATTAAAATGGGTTTCACTGTGAA 1440
1441  TGCGTGACAATAAGATATTCCCTTGTTCCTAAAACTTTATATCAGTTTATTGGATGTGGG 1500
1501  TTTtTCACATTTAAGATAATTATGGCTCTTTTCCTAAAAAATAAAATATCTTTCTAAAAA 1560
1561  AAAAAAAA  1568
```

FIG. 12

CTAGATGAGGCAGATATAAGAGTCA 25

TGGAAAAAAGGACAGAGAAAAAAAA 50

CAGACAAATCAGTTGTCAGTATCCA 75

TGGCCTCTGATTCTGTCTCAACCAT 100

GAAACAGAAGTGACACATATAC 122

CTGCTAAAAG

FIG. 13

AAC	TCA	GTC	CCT	TTC	CTT	CCA	AAA	TCT	27
CCA	ACA	TGC	GGT	CCT	TAT	TCT	GAT	CAA	54
TTT	CTG	GGA	GCA	TTT	TCT	GCA	TAT	TGA	81
CCT	TGC	TTT	GTT	GAA	AAA	GTT	CTG	TTA	108
ACC	ATT	CTC	GAT	CTT	GTA	ATT	TAG	CTA	135
ACT	GCT	GAA	GAC	AAA	GTA	GGA	AGA	GAG	162
GAA	AAT	GGG	TGC	CAC	TTT	CTA	GTG	GTT	189
GAG	CTA	GTT	CTG	AAA	TGC	TCA	CCA	GCT	216
CTG	AAA	TGA	TGG	CAC	GAG	CTG	CAA	ACT	243
GTG	CTA	AAT	AGC						

• = ?

FIG. 14

```
                                                          M  Q  P
   1  GGCTATAAGCGCACGGCCTCGGCGACCCTCTCCGACCCGGCCGCCGCCATGCAGCCC    60
      S  S  L  L  P  L  A  L  C  L  L  A  A  P  A  S  A  L  V  R
  61  TCCAGCCTTCTGCCGCTCGCCCTCTGCCTGCTGGCTGCACCCGCCTCCGCGCTCGTCAGG   120
      I  P  L  H  K  F  T  S  I  R  R  T  M  S  E  V  G  G  S  V
 121  ATCCCGCTGCACAAGTTCACGTCCATCCGCCGGACCATGTCGGAGGTTGGGGGCTCTGTG   180
      E  D  L  I  A  K  G  P  V  S  K  Y  S  Q  A  V  P  A  V  T
 181  GAGGACCTGATTGCCAAAGGCCCCGTCTCAAAGTACTCCCAGGCGGTGCCAGCCGTGACC   240
      E  G  P  I  P  E  V  L  K  N  Y  M  D  A  Q  Y  Y  G  E  I
 241  GAGGGGCCCATTCCCGAGGTGCTCAAGAACTACATGGACGCCCAGTACTACGGGGAGATT   300
      G  I  G  T  P  P  Q  C  F  T  V  V  F  D  T  G  S  S  N  L
 301  GGCATCGGGACGCCCCCCCAGTGCTTCACAGTCGTCTTCGACACGGGCTCCTCCAACCTG   360
      W  V  P  S  I  H  C  K  L  L  D  I  A  C  W  I  H  H  K  Y
 361  TGGGTCCCCTCCATCCACTGCAAACTGCTGGACATCGCTTGCTGGATCCACCACAAGTAC   420
      N  S  D  K  S  S  T  Y  V  K  N  G  T  S  F  D  I  H  Y  G
 421  AACAGCGACAAGTCCAGCACCTACGTGAAGAATGGTACCTCGTTTGACATCCACTATGGC   480
      S  G  S  L  S  G  Y  L  S  Q  D  T  V  S  V  P  C  Q  S  A
 481  TCGGGCAGCCTCTCCGGGTACCTGAGCCAGGACACTGTGTCGGTGCCCTGCCAGTCAGCG   540
      S  S  A  S  A  L  G  G  V  K  V  E  R  Q  V  F  G  E  A  T
 541  TCGTCAGCCTCTGCCCTGGGCGGTGTCAAAGTGGAGAGGCAGGTCTTTGGGGAGGCCACC   600
      K  Q  P  G  I  T  F  I  A  A  K  F  D  G  I  L  G  M  A  Y
 601  AAGCAGCCAGGCATCACCTTCATCGCAGCCAAGTTCGATGGCATCCTGGGCATGGCCTAC   660
      P  R  I  S  V  N  N  V  L  P  V  F  D  N  L  M  Q  Q  K  L
 661  CCCCGCATCTCCGTCAACAACGTGCTGCCCGTCTTCGACAACCTGATGCAGCAGAAGCTG   720
      V  D  Q  N  I  F  S  F  Y  L  S  R  D  P  D  A  Q  P  G  G
 721  GTGGACCAGAACATCTTCTCCTTCTACCTGAGCAGGGACCCAGATGCGCAGCCTGGGGGT   780
      E  L  M  L  G  G  T  D  S  K  Y  Y  K  G  S  L  S  Y  L  N
 781  GAGCTGATGCTGGGTGGCACAGACTCCAAGTATTACAAGGGTTCTCTGTCCTACCTGAAT   840
      V  T  R  K  A  Y  W  Q  V  H  L  D  Q  V  E  V  A  S  G  L
 841  GTCACCCGCAAGGCCTACTGGCAGGTCCACCTGGACCAGGTGGAGGTGGCCAGCGGGCTG   900
      T  L  C  K  E  G  C  E  A  I  V  D  T  G  T  S  L  M  V  G
 901  ACCCTGTGCAAGGAGGGCTGTGAGGCCATTGTGGACACAGGCACTTCCCTCATGGTGGGC   960
      P  V  D  E  V  R  E  L  Q  K  A  I  G  A  V  P  L  I  Q  G
 961  CCGGTGGATGAGGTGCGCGAGCTGCAGAAGGCCATCGGGGCCGTGCCGCTGATTCAGGGC  1020
      E  Y  M  I  P  C  E  K  V  S  T  L  P  A  I  T  L  K  L  G
1021  GAGTACATGATCCCCTGTGAGAAGGTGTCCACCCTGCCCGCGATCACACTGAAGCTGGGA  1080
      G  K  G  Y  K  L  S  P  E  D  Y  T  L  K  V  S  Q  A  G  K
1081  GGCAAAGGCTACAAGCTGTCCCCAGAGGACTACACGCTCAAGGTGTCGCAGGCCGGGAAG  1140
      T  L  C  L  S  G  F  M  G  M  D  I  P  P  P  S  G  P  L  W
1141  ACCCTCTGCCTGAGCGGCTTCATGGGCATGGACATCCCGCCACCCAGCGGGCCACTCTGG  1200
      I  L  G  D  V  F  I  G  R  Y  Y  T  V  F  D  R  D  N  N  R
1201  ATCCTGGGCGACGTCTTCATCGGCCGCTACTACACTGTGTTTGACCGTGACAACAACAGG  1260
      V  G  F  A  E  A  A  R  L  *
1261  GTGGGCTTCGCCGAGGCTGCCCGCCTCTAGTTCCCAAGGCGTCCGCGCGCCAGCACAGAA  1320
1321  ACAGAGGAGAGTCCCAGAGCAGGAGGCCCCTGGCCCAGCGGCCCCTCCCACACACACCCA  1380
1381  CACACTCGCCCGCCCACTGTCCTGGGCGCCCTGGAAGCCGGCGGCCCAAGCCCGACTTGC  1440
1441  TGTTTTGTTCTGTGGTTTTCCCCTCCCTGGGTTCAGAAATGCTGCCTGCCTGTCTGTCTC  1500
1501  TCCATCTGTTTGGTGGGGGTAGAGCTGATCCAGAGCACAGATCTGTTTCGTGCATTGGAA  1560
1561  GACCCCACCCAAGCTTGGCAGCCGAGCTCGTGTATCCTGGGGCTCCCTTCATCTCCAGGG  1620
1621  AGTCCCCTCCCCGGCCCTACCAGCGCCCGCTGGGCTGAGCCCCTACCCCACACCAGGCCG  1680
1681  TCCTCCCGGGCCCTCCCTTGGAAACCTGCCCTGCCTGAGGGCCCCTCTGCCCAGCTTGGG  1740
1741  CCCAGCTGGGCTCTGCCACCCTACCTGTTCAGTGTCCCGGGCCCGTTGAGGATGAGGCCG  1800
1801  CTAGAGGCCTGAGGATGAGCTGGAAGGAGTGAGAGGGGACAAAACCCACCTTGTTGGAGC  1860
1861  CTGCAGGGTGGTGCTGGGACTGAGCCAGTCCCAGGGGCATGTATTGGCCTGGAGGTGGGG  1920
1921  TTGGGATTGGGGGCTGGTGCCAGCCTTCCTCTGCAGCTGACCTCTGTTGTCCTCCCCTTG  1980
1981  GGCGGCTGAGAGCCCCAGCTGACATGGAAATACAGTTGTTGGCCTCCGGCCTCCCCTC  2038
```

FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11598

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 69.1, 320.1; 536/23.5, 24.31, 24.5; 530/350, 387.1; 424/93.2, 130.1; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: apoptosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,542,096 (LEDER) 17 September 1985, entire document.	15,16
Y	US, A, 5,015,570 (SCANGOS et al.) 14 May 1991, entire document.	15,16
Y	US, A, 4,798,787 (McCORMICK et al.) 17 January 1989, entire document.	17
Y	Proc. Natl. Acad. Sci. USA, Volume 84, issued March 1987, N.V. Katre et al., " Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model", pages 1487-1491, entire document.	7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 JANUARY 1995	17 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer LORRAINE M. SPECTOR
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

International application No.
PCT/US94/11598

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Proc. Natl. Acad. Sci. USA, Volume 82, issued August 1985, P.L. Faust et al., "Cloning and sequence analysis of cDNA for human cathepsin D", pages 4910-4914, entire document.	1,3,4,6,9-12,14,15,18-20 5,7
A,P	Cell, Volume 75, issued 03 December 1993, C. Abbadie et al., "High levels of c-rel expression are associated with programmed cell death in the developing avian embryo and in bone marrow cells in vitro", pages 899-912, entire document.	1,3-7,9-20
A,P	Cell, Volume 75, issued 19 November 1993, M. Miura et al., "Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3", pages 653-660, entire document.	1,3-7,9-20
Y	Molecular and Cellular Biology, Volume 11, No. 8, issued August 1991, G.P. Owens et al., "Identification of mRNAs associated with programmed cell death in immature thymocytes", pages 4177-4188, entire document.	1,3-7,10-14,18-20
A	Leukemia Research, Volume 17, No. 8, issued August 1993, J. Moore et al., "Mutant *H-Ras* over-expression inhibits a random apoptotic nuclease in myeloid leukemia cells", pages 703-709, entire document.	1,3-7,10-20
Y	Science, Volume 252, issued 05 April 1991, L.P. Deiss et al., "A genetic tool used to identify thioredoxin as a mediator of a growth inhibitory signal", pages 117-120, entire document.	5
A	J. Biological Chemistry, Volume 268, No. 7, issued 05 March 1993, P. Wong et al., "Genomic organization and expression of the rat TRPM-2 (clusterin) gene, a gene implicated in apoptosis", pages 5021-5031, entire document.	1,3-7,10-20
A	J. Biological Chemistry, Volume 268, No. 15, issued 25 May 1993, N. Itoh et al., "A novel protein domain required for apoptosis. Mutational analysis of human *Fas* antigen", pages 10932-10937, entire document.	1,3-7,10-20
A	Experimental Hematology, Volume 21, No. 11, issued October 1993, T. Nakano et al., "Transfection of interferon-γ gene in a mouse bone marrow stromal preadipocyte cell line causes apoptotic cell death", pages 1498-1503, entire document.	1,3-7,10-15, 20

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Molecular Neuroscience, Volume 4, No. 4, issued April 1993, S.R. D'Mello et al., "SGP2, ubiquitin, 14K lectin and RP8 mRNAs are not induced in neuronal apoptosis", pages 355-358, entire document.	1,3-7,10-20
A	Nature, Volume 352, issued 25 July 1991, E. Yonish-Rouach et al., "Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin 6", pages 345-347, entire document.	1,3-7,10-20

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/68; C07H 21/04; G01N 33/53; C12N 15/00, 15/12; C07K 1/00, 16/18; A61K 38/02, 38/19, 39/395; 48/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 69.1, 320.1; 536/23.5, 24.31, 24.5; 530/350, 387.1; 424/93.2, 130.1; 514/2

INTERNATIONAL SEARCH REPORT	International application No. PCT/US94/11598

Box I Observations where certain claims were found unsearchable (Continuation of item 1 f first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2 and 8
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

these claims recite particular nucleotide and/or amino acid sequences. As no CRF has been submitted in this case, no search could be performed.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.